EFFECTS OF A NON-STEROIDAL AROMATASE INHIBITOR ON HUMAN OVARIAN FUNCTION

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By

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ABSTRACT

Introduction: Ovarian folliculogenesis occurs in a wave-like pattern of growth during the menstrual cycle. A better understanding of the role of estrogen in folliculogenesis may lead to the development of better contraceptive and fertility protocols. Aromatase inhibitors cause a transient decline in natural estrogen production. Multiple small doses of an aromatase inhibitor have been used during fertility therapy to induce dominant follicle growth and ovulation.

Objectives: Our objectives were to elucidate the effects of a single 20 mg dose of an aromatase inhibitor (Letrozole/Femara™) on folliculogenesis and test the hypothesis that an AI administered during defined times of the follicular phase or immediately after ovulation would result in atresia of the extant dominant follicle and initiate new wave emergence.

Methods: Healthy, reproductive age, female volunteers not taking hormonal contraceptives were recruited (n=41). Ultrasound examinations began on day 4 of the menstrual cycle (day 1=first day of menses). Volunteers were randomized into 1 of 4 groups: treatment at a follicle diameter of 1) 12 mm, 2) 18 mm, 3) the first day following ovulation, or 4) control. Serial ultrasonography and phlebotomy were performed until ovulation in the subsequent cycle. Differences were analyzed using t-test, ANOVA, repeated measures ANOVA, or Kruskal-Wallis ANOVA where appropriate.

Results: The dominant follicle in all treatment groups ovulated. There were no differences among experimental groups for peak follicle diameter, follicular growth rate, endometrial thickness at ovulation, or inter-ovulatory interval. Maximum FSH concentrations in the 12 mm, 18 mm and post-OV groups were 12.58 ± 1.41 mIU/mL, 18.62 ± 2.27 mIU/mL, 12.38 ± 1.10 mIU/mL, respectively. Maximum LH concentrations in the 12 mm, 18 mm and post-OV groups were 16.20 ±2.06 mIU/mL, 40.43 ± 4.32 mIU/mL, 16.34 ± 2.59 mIU/mL,

respectively. The 18 mm group had higher FSH and LH concentrations ($P < 0.02$) compared to the 12 mm and post-OV groups.

Conclusions: Administration of a single 20 mg dose of an aromatase inhibitor at defined times of the menstrual cycle did not induce dominant follicle regression or new wave emergence. Treatment resulted in continued follicle development, a transient decrease in E_2 levels and elevated circulating FSH and LH concentrations. The failure of an aromatase inhibitor to interrupt dominant follicle development suggests a compensatory mechanism for the acute drop in E_2 that may involve increased LH and FSH levels.

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DEDICATION

To my mom, Patricia Allaway.

"Lucy" we have been through many things together in the last 6 years. You are the strongest woman I have ever known. You have taught me how to persevere and prepared me to face the challenges of life with love and hope. You continue to be my inspiration and unconditional supporter.

Thank you for believing in me when I could not see the forest through the trees to believe in myself. Your undying faith in me has allowed me to follow what I love. Thank you for encouraging me to follow where the adventure leads. No matter how far away the adventure takes me I know you are there with me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A-mode	amplitude mode
aa	amino acids
AI	aromatase inhibitor
AMH	anti-mullerian hormone
ATP	adenosine triphosphate
B-mode	brightness mode
CA	corpus albicans
cAMP	cyclic adenosine monophosphate
CH	corpus haemorrhagicum
CL	corpus luteum
cm	centimeter
d	day
DF	dominant follicle
E_2	estradiol
EC	emergency contraception
EE	ethinyl estradiol
FDA	USA Food and Drug Administration
FSH	follicle stimulating hormone
FSHr	follicle stimulating hormone receptor
GC	granulosa cell
h	hour
HAF	haemorrhagic anovulatory follicle
hCG	human chorionic gonadotropin
Hg	mercury
IGF	insulin-like growth factor
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
IGFBP-4	insulin-like growth factor binding protein 4
IMI	inter-menstrual interval
IOI	interovulatory interval
IUD	intrauterine device
kDa	kilo dalton
L	litre
LDL	low density lipoprotein
LH	luteinizing hormone
LHr	luteinizing hormone receptor
LNG	levonorgestrel
LUF	luteinized unruptured follicle
M-mode	motion mode
mg	milligram
min	minute

mIU	milli International Units
mL	millilitre
mm	millimeter
mRNA	messenger ribonucleic acid
NADP+	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ng	nanogram
OC	oral contraception
OV	ovulation
P_4	progesterone
PGE_2	prostaglandin E2
$PGF_{2\alpha}$	prostaglandin F2alpha
pixel	picture element
pmol	pico mole
sec	second
TVS	transvaginal ultrasonography
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor
WE	wave emergence
ZP	zona pellucida
μm	micrometer

Chapter 1: General Introduction

The ovary is the "master" gland of the female reproductive system. In this role, it has dual endocrine and exocrine functions that are involved in ovarian follicle growth, ovulation (OV) and regression. Menses is the external sign of reproductive cyclicity in humans. The traditional model of the human menstrual cycle has two phases, follicular and luteal, based on menses. The follicular phase is defined as the stage of the menstrual cycle that begins on the first day of menstrual bleeding. During the mid-follicular phase a single "dominant" follicle is selected from the recruited cohort for preferential growth and ovulation. The selected follicle secretes increasing amounts of estradiol (E_2) as it grows to pre-ovulatory size. The luteal phase is defined as the stage of the menstrual cycle immediately following pre-ovulatory follicle collapse at OV which comprises the formation of the corpus luteum (CL), and secretion of progesterone (P_4). The luteal phase ends the day prior to the first day of the next menses.

The dynamics of human ovarian function were not well understood prior to the introduction of ultrasonography for the detailed study of follicular growth in domestic animals. Incorporation of high-resolution ultrasonography in ovarian function research, as used in animal models, has allowed the use of OV as the definitive endpoint of defining cyclicity. Cyclicity in this sense is termed an inter-ovulatory interval (IOI). This development has facilitated comparative research between species. Increasing evidence from ultrasonography indicates that multiple waves of antral follicles develop throughout the human menstrual cycle. Waves of ovarian follicular development in women are comparable to waves documented in several other domestic animal species; however, species differences do exist (1).

Two or three waves (or cohorts) of follicles develop through out an IOI. The final wave in an IOI is ovulatory which is consistent across species. The recruitment of follicles into follicular waves is initiated by a rise in the circulating concentration of follicle stimulating hormone (FSH). Following the initial rise in FSH, a decline in FSH occurs and plays a critical role in dominant follicle selection (2-6). The growth profile of the dominant follicle begins to diverge from subordinate follicles in the ovulatory wave at approximately 10 mm in diameter (3, 4, 7, 8). The dominant follicle produces inhibin B and E_2 that suppresses the continued growth of subordinate follicles within the same wave and the emergence of a new wave through inhibition of pituitary FSH secretion (2, 9-11).

Basal luteinizing hormone (LH) concentrations induce production of androgens in thecal cells that become the substrate for granulosa cells (GC) production of E_2 (discussed in 2.3.2)(12-14). Estradiol production and formation of LH receptors (LHr) on the GC by the selected follicle is important in its continued growth. The formation of LHr shifts the dominant follicle from FSH to LH receptiveness in the decreasing FSH environment of follicle selection (15-17). The dominant follicle in women reaches pre-ovulatory size at approximately 22 mm in diameter (8, 18-22). Estradiol production reaches a threshold level and peaks providing positive feedback on the hypothalamus/pituitary axis and stimulates the pre-ovulatory surge in LH (22, 23). Circulating P_4 concentrations begin to rise after the E_2 peak and prior to the LH surge (22, 24-26). Ovulation normally occurs within 24 hours (h) of the LH surge reaching its peak concentration (27). Control of follicle growth and OV are the focus of fertility therapies and contraception.

Emergency contraception (EC) was developed empirically with the intent of suppressing follicular development, OV, or the ability of a conceptus to implant in the uterus (28-30). Modern EC has its roots in 1920s veterinary medicine (31, 32). Estrogenic

ovarian extracts were injected intramuscularly in dogs and were shown to interfere with pregnancy (31, 32). In the 1970s, research began into the use of combined (estrogen and progestin) and progestin-only hormonal EC (31, 32). Combined and progestin-only hormonal formulations are the most common EC currently in use; however, the response of the ovaries to hormonal EC is unpredictable. Emergency contraception does not inhibit OV in the presence of a pre-ovulatory dominant follicle (33-36). The inability of EC to prevent OV is problematic given the routine clinical use of the medication. It is therefore necessary to re-examine the effects of E_2 on follicle growth.

Aromatase inhibitors (AI) prevent the enzymatic conversion of androgens to estrogens and subsequently lower the serum estrogen concentration (37-41). When AI are administered before selection as part of fertility treatments, pre-ovulatory sized follicles are typically observed 10 to 12 days later (42-49). The inhibition of aromatase prevents estrogen production and releases the hypothalamic/pituitary axis from the negative feedback of E_2. The release results in an increase in FSH secretion and stimulation of ovarian follicle growth (44, 45, 49-51). We theorize that if AI were administered after follicle selection the acute E_2 deprivation would initiate atresia of the dominant follicle in the extant wave. Estradiol deprivation will release negative feedback on FSH and result in the emergence of a new follicular wave without an ovulatory event occurring. We postulate that information regarding the mechanisms of follicular suppression and atresia could be used to develop new contraceptive technologies.

The research in this thesis is focused on the physiologic and endocrinologic effects of a single, large dose of an AI on ovarian follicle and endometrial development at biologically important times of the natural menstrual cycle. We hypothesized that a temporary drop in E_2 would lead to atresia of the dominant follicle and a shortened interval

to menstruation. The literature review presents a synopsis of the available data on ovarian folliculogenesis during natural cycles and administration of EC and AI. In addition, the principles of ultrasound imaging are reviewed to provide a brief explanation of how the convergence of research and clinical techniques was brought to bear on this important clinical and physiological question.

Chapter 2: Literature Review

2.1. Human Female Reproductive Anatomy

2.1.1. Human Ovarian Anatomy

The ovaries are a pair of dull white, almond shaped structures that lie on either side of the uterus (52). Ovaries develop as indifferent gonadal thickenings along the ventral cranial mesonephros (genital ridge) in the lumbar region during embryonic development (53). The germ cells migrate from the yolk sac to the genital ridge during week 6 of human gestation (53). The ovaries, complete with the complement of germ cells, gradually descend to the lesser pelvis in early fetal life until they lie on either side of the uterus. In adults, the ovaries are held in place by three ligaments. The ovaries are attached to the posterior-superior aspect of the broad ligament, postero-inferior to the fallopian tube (52, 54). Ovarian and suspensory ligaments hold the ovaries in the peritoneal space near the fimbriated end of the fallopian tube (52).

The size of ovaries change in relation to the stage of follicular development and a woman's age. However, typically normal healthy ovaries do not usually exceed 4 x 3 x 2 centimetres (cm) (52). A flattened layer of cuboidal cells called the germinal epithelium covers the outside of the ovary (54). Immediately beneath the germinal epithelium is the tunica albuginea, a condensed, delicate layer of connective tissue (54). The body of the ovary is broken into the outer, thick cortex and the inner medulla. The cortex contains follicles and corpora lutea at different stages of development and regression (54). The medulla of the ovary does not contain any follicles (54). The ovarian blood vessels, lymphatic vessels and nerves enter the ovary through the hilum and terminate in the medulla (54).

The suspensory ligament contains the ovarian vessels and nerves which enter the ovary at the hilus (53, 54). The ovarian arteries branch off the abdominal aorta and supply

the ovaries, fallopian tubes, labium majus, and the inguinal region (54). Medullary ovarian veins emerge as a plexus from each ovarian hilus and then form ovarian veins. The ovarian veins branch into the inferior vena cava on the right side and the renal vein on the left. The ovarian nerves follow the ovarian arteries to the ovaries and the fallopian tubes (54). The ovary only has sympathetic innervation which is supplied by post-ganglionic fibers from the ovarian plexus. The sympathetic fibres of the ovarian plexus emerge from the tenth and eleventh thoracic spinal segment and are vasoconstrictory.

2.1.2. Human Uterine Anatomy

The human uterus is a hollow, pear-shaped, muscular organ lying posterior to the urinary bladder and anterior to the rectum (52, 55). The uterus is an average of 7.5 cm in length, 2.5 cm in depth and 5 cm in width at its widest point (52). It can be anatomically divided into the fundus, body, and cervix (54). The body of the uterus narrows gradually from the fundus to the internal os of the cervix (54). The fallopian tubes pass through the lateral uterine horns at the top of the uterine body at the level of the fundus, forming a connection from each ovary to the uterus.

The wall of the uterus consists of 3 layers, the perimetrium, myometrium, and endometrium. The perimetrium is the outer covering of the uterus (54). The myometrium is the thick muscular layer consisting of interlaced longitudinal and circular muscle fibers (54). The muscle fibers of the myometrium are arranged in 3 layers: external, middle, and internal (54). Blood vessels, lymph vessels, and nerves are intermixed in the muscular layers (54). The myometrium also contain spiral arterioles and tube-like uterine glands (54). The spiral arterioles and uterine glands extend into the endometrium.

The endometrium is the inner mucosal layer of the uterus. It is composed of non-ciliated, columnar epithelium and is broken into the stratum basalis and functionalis (54).

The stratum basalis is the subepithelial layer of the endometrium. It is a nucleated, highly cellular form of connective tissue capable of further development and contains blood vessels, numerous lymphatic spaces, and uterine glands (54). The stratum functionalis is subdivided into the stratum spongiosum and stratum compactum layers. The stratum functionalis is shed during menstruation and thereafter regenerated by the stratum basalis (54). The endometrium thickens during the early follicular phase, under the influence of dominant follicle E_2 production (56). The endometrium reaches approximately 6-10 mm in thickness at the end of the follicular phase and 7-14 mm by the end of the luteal phase (56). During the late luteal phase of the menstrual cycle the tall columnar cells become frayed and worn at the luminal aspect of the cell (54). Decomposition begins at the luminal edge of the endometrium and extends to the deeper stratum spongiosum (54). Blood and the necrotic epithelium are shed into the lumen of the uterus and are discharged via the cervix during menses.

The uterus is connected to the surrounding tissue by multiple ligaments. The anterior ligament connects the uterus to the bladder at the junction of the cervix and uterine body (54). The posterior ligament connects the posterior fornix of the vagina (fibromuscular tube connecting the uterus to the external genitalia) to the front of the rectum (54). The broad ligaments pass from the sides of the uterus to the lateral walls of the pelvis and also hold the fallopian tubes and ovaries in position (54). Two uterine branches of the internal iliac arteries, which run through the broad ligaments, are the main arterial supply of the uterus.

2.2. Human Ovarian Dynamics

2.2.1. Pre-Antral Follicle Development

Oogenesis, the formation and development of an ovum, occurs during fetal life. Primordial germ cells migrate from the yolk sac to the genital ridge in the developing embryo

to form oogonia. The germ cells not exposed to stem cell growth factor and basic fibroblast growth factor during transit to the genital ridge from the yolk sac undergo apoptosis (57). In the genital ridge the germ cells form oogonial nests and undergo rapid mitosis (58). Somatic cells from the mesonephros surround oogonia in the genital ridge to form primordial follicles (58). Oocytes in primary follicles cease mitotic divisions and begin meiotic division (58). Meiosis of oocytes in primordial follicles arrests in prophase I (58). The population of primordial follicles in the ovaries constitutes the ovarian follicular reserve (59, 60). Oogonia not enveloped by somatic cells degenerate (57, 58), thus regulating the endowment of the ovarian reserve.

Female mammals are born with their complete complement of oocytes in the ovarian reserve (59). Our current understanding is that female mammals are incapable of producing more oocytes during reproductive life span (59). The total number of germ cells reaches approximately 6,800,000 at 5 months of fetal development the mitotic divisions (59). Once the germ cells form primordial follicles and enter the process of meiosis the total number of follicles in the ovaries declines progressively. At birth, the population of primordial follicles in the ovary is approximately 1,000,000 and at menarche the ovarian reserve consists of approximately 270,000 to 470,000 follicles (59). After menarche, there is a cyclic depletion of approximately 100 follicles from the ovarian reserve each month(61-64). The depletion of follicles from the ovarian reserve results from the entrance of follicles into waves (aka cohorts) of development. By menopause the ovarian reserve reaches 100 to 1000 primordial follicles (62).

Primordial follicles are comprised of a single layer of somatic cells surrounding primary oocytes. Primary oocytes are germ cells arrested in the diplotene stage of meiotic prophase I at a size of 32 micrometre (μm) until they are stimulated to resume meiosis

during an ovarian cycle (65, 66). The diameter of the oocyte initially increases from approximately 30 μm in primary follicles until it reaches approximately 140 μm in diameter in pre-ovulatory follicles (67). The morphologic changes associated with each stage of pre-antral, as well as antral, follicle growth are summarized (Table 2.1).

Initiation of follicle growth into a pool of growing primordial follicles is a multiphasic, continual process regulated by biological molecules that starts during fetal development and continues until menopause (66, 67). The phases of follicle growth, development and the length of time required to transition from a primordial follicle to an ovulatory follicle are illustrated (Figure 2.1).

The first changes in primordial follicular growth are recognized as the differentiation of spindle-shaped GC into cuboidal cells (68) and the formation of the zona pellucida (ZP), a protective glycoprotein layer around the oocyte secreted by GC (69). The cuboidal GC proliferate and differentiate into cumulus cells surrounding the oocyte and mural cells lining the interior aspect of the follicle (70). As preantral follicles increase in diameter, the surrounding stromal tissue differentiates into two layers: the theca interna and theca externa (Table 2.1)(60). The blood supply in the thecal layer is typically provided by one or two arterioles which terminate in a wreath-like pattern of capillaries surrounding the follicle and adjacent to the basal lamina (68, 71, 72). At a diameter of 0.2 millimeter (mm) an antrum develops within the growing follicles (65, 73). Recruitment of antral follicles into a growing phase occurs at regular intervals during the menstrual cycle (3, 74-76).

Table 2.1: Classification of follicles and morphological changes associated with stages of follicular development in the human ovary (modified from Gougeon, Endocrine Reviews, 1996)(77).

Phase of Development	Stage of Development	Morphological Characteristics
Resting Follicle	Primordial Follicle	• 35 μm in diameter • single layer of flattened GC
	Intermediary Follicle	• 38 μm in diameter • single layer of flattened and cuboidal GC
Early Growing	Primary Follicle	• 46 μm in diameter • single layer of cuboidal GC • zona pellucida develops
	Secondary Follicle	• 77 μm in diameter • > 1 layer of cuboidal GC • GC express FSH, E_2, and androgen receptors • Theca interna express Lr • Theca interna and externa differentiate • follicular vascular and lymphatic systems differentiate • 99% of follicles enter atresia
Pre-Antral	Class 1	• 0.1-0.2 mm in diameter • 6 x 10^2 GC • 2-cell-2 gonadotropin hormone synthesis • no antrum • 24% of follicles enter atresia
Early Antral	Class 2	• 0.2-0.4 mm in diameter • 3-5 x 10^3 GC • cumulus oophorus develops • antrum development commences • 35% of follicles enter atresia
	Class 3	• 0.4-0.9 mm in diameter • 1.5 x 10^4 GC • small antrum • 15% of follicles enter atresia
	Class 4	• 0.9-2.0 mm in diameter • 75 x 10^4 GC • medium antrum • 24% of follicles enter atresia
Recruited	Class 5	• 2.0-5.0 mm in diameter • 37 x 10^5 GC • medium antrum • 58% of follicles enter atresia
Selected	Class 6	• 5.0-10.0 mm in diameter • 19 x 10^6 GC • medium antrum • aromatase activity detected in GC • 77% of follicles enter atresia
Early Pre-Ovulatory	Class 7	• 10.0-16.0 mm in diameter • 94 x 10^6 GC • large antrum
Pre-Ovulatory	Class 8	• 16.0 - 24 mm in diameter • 47-60 x 10^6 GC • ovulation occurs at the end of the phase

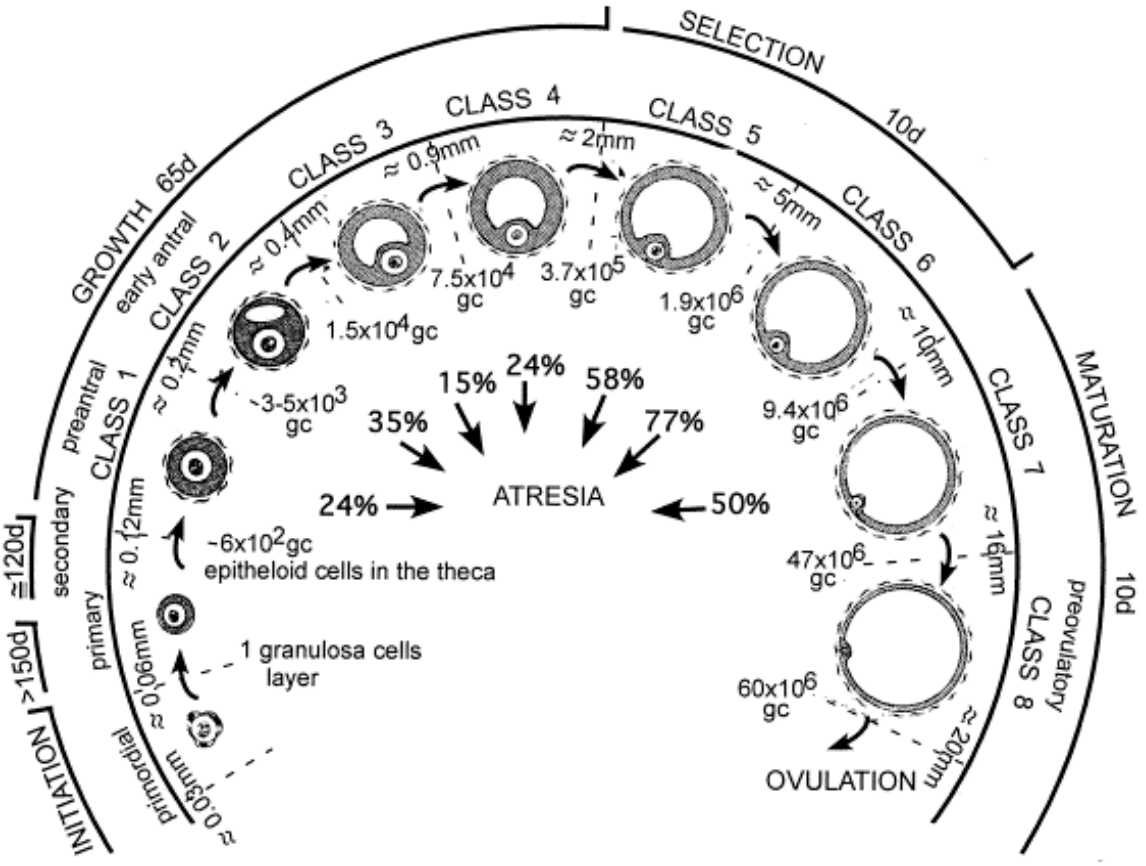

Figure 2.1: Classification of follicles development stages in the human ovary. Level of atresia and number of granulosa cells occurring in class 1 to 8 growing follicles. The approximate follicle diameter at the transition between classes and duration of growth at each stage are indicated (from Gougeon, Human Reproduction, 1986)(20).

2.2.2. Antral Follicle Development

Follicle wave patterns of development, first described in domestic animals, (i.e., bovine and equine) have been documented in several other mammalian species, including women (18, 78-85). Comparative studies have shown similarities in ovarian follicular wave dynamics between monovular domestic farm animals and humans (86-88). Follicular waves are defined as the synchronous growth of cohorts of follicles from which one or more follicles may be selected for preferential growth (89). The follicles recruited into the wave are similar, but not identical, diameters (3, 81, 88, 90). Most women exhibit 2 waves of follicle growth (68%) while the remaining 32% exhibit 3 waves of follicle growth (18).

Antral follicular development can be characterized over an inter-ovulatory interval (IOI; i.e., from OV to OV) or across the menstrual cycle (i.e, from menses to menses; an inter-menstrual interval; IMI). The advantage of evaluating follicular dynamics over an IOI is that it allows characterization of ovarian function across the entire ovarian cycles, regardless of uterine function. Each IOI (or corresponding menstrual cycle) is comprised of 2 or 3 waves of follicle development. (Figure 2.2a-f)(1, 3, 18, 90). Emergence of each follicular wave during the IOI in women is preceded by an elevation in circulating FSH (3), consistent with previous reports in domestic animals (2, 3, 91, 92). Major waves are defined as those in which a dominant follicle is selected at 10 mm in diameter to continue growth while all other follicles undergo atresia (3, 80). In minor waves selection of a dominant follicle is not manifest and no follicles grow > 10 mm in diameter (3, 80). The final wave of the IOI is a major ovulatory wave, while all preceding waves, major or minor, are anovulatory (3, 86). The earlier wave emergence, selection, and pre-ovulatory surge in 2 wave cycles leads to a shorter average IOI and corresponding menstrual cycle compared to 3 wave cycles (3).

2.2.2.1. Follicluar Dynamics

The growth of the antral follicle waves consists of up to 4 stages, depending on whether the wave is major or minor, ovulatory or anovulatory. The 4 stages are: 1) wave emergence (also referred to as recruitment of the follicular cohort); 2) selection 3) pre-ovulatory follicle growth; and, 4) OV (66). Each of these important physiologic events is discussed below.

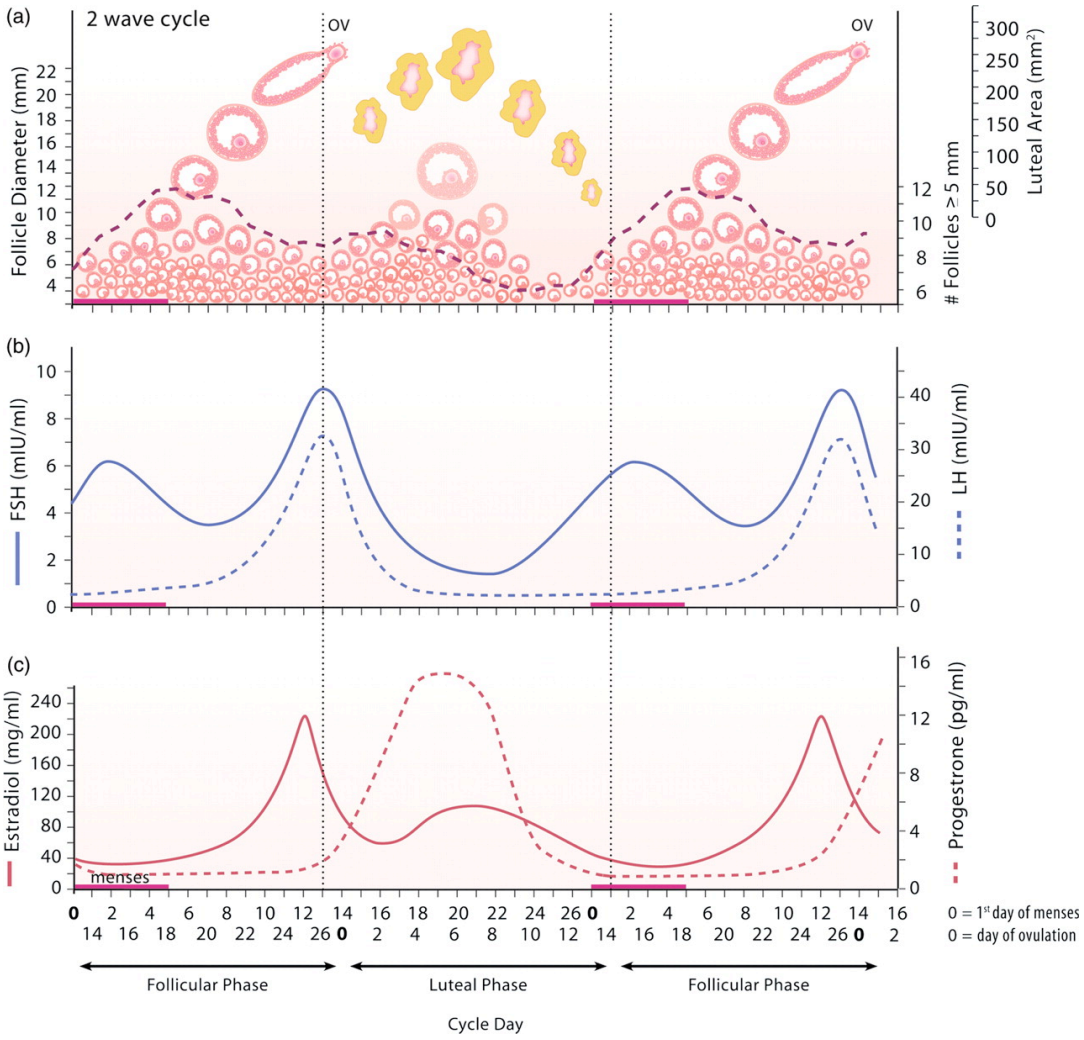

Figure 2.2 a-c: Morphologic and endocrinologic changes associated with 2 waves of follicle development during the menstrual cycle. Data encompass one complete menstrual cycle and one complete inter-ovulatory interval. Vertical lines indicate the days of wave emergence, follicles are shown in pink, and the corpus luteum is in yellow. Major anovulatory waves are ghosted due to detection in some but not all women prior to the ovulatory wave. (From Baerwald, et. al, Human Reproduction Update, 2012)(1).

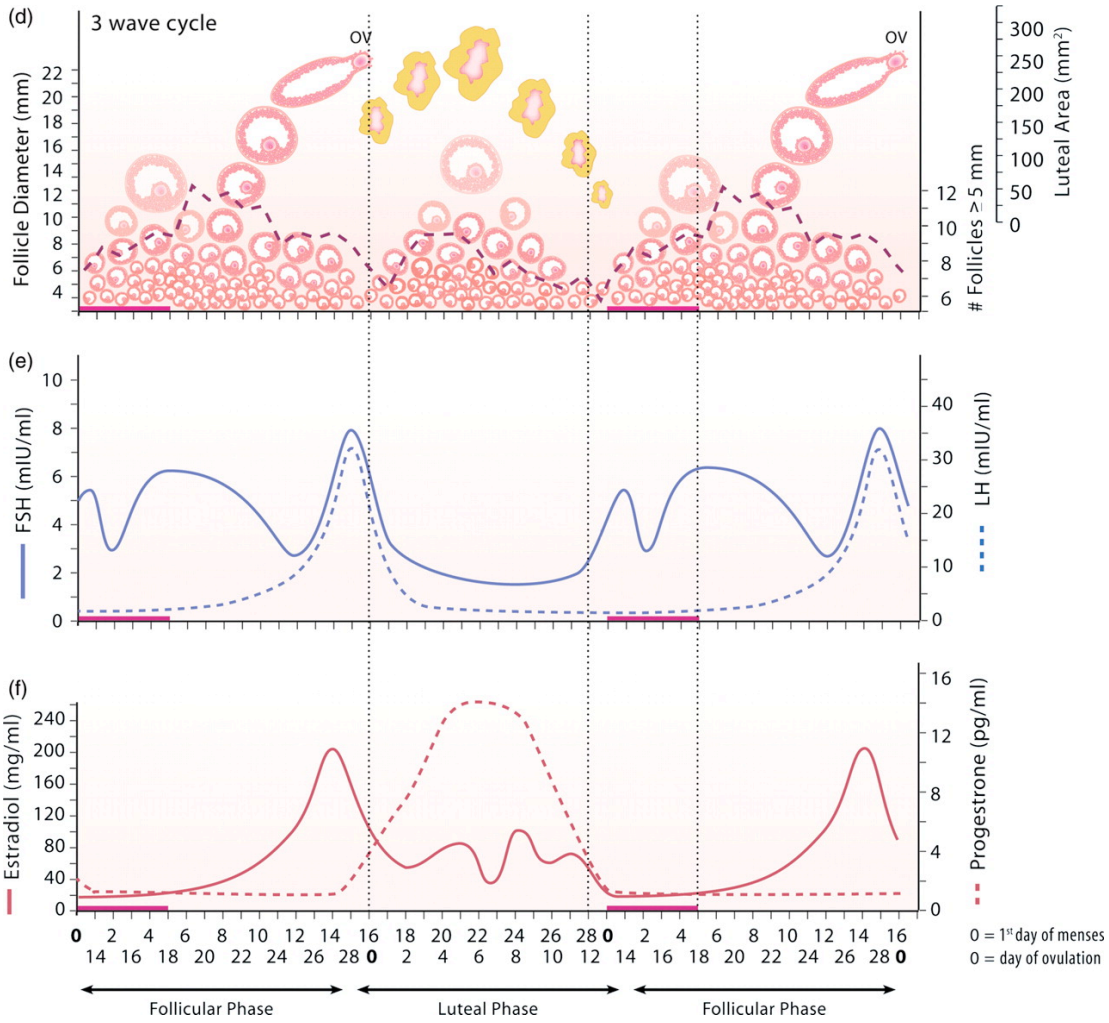

Figure 2.2 d-f: Morphologic and endocrinologic changes associated with 3 waves of follicle development during the menstrual cycle. Data encompass one complete menstrual cycle and one complete inter-ovulatory interval. Vertical lines indicate the days of wave emergence, follicles are shown in pink, and the corpus luteum is in yellow. Major anovulatory waves are ghosted due to detection in some but not all women prior to the ovulatory wave. (From Baerwald, et. al, Human Reproduction Update, 2012)(1).

2.2.2.1.1. Wave Emergence

A cohort of 3 to 20 antral follicles between 2.0-5.0 mm in diameter is recruited to grow at regular intervals over the menstrual cycle (3, 8, 68, 93). Emergence of the follicular wave is preceded by an elevation in circulating FSH (3). A rise in FSH levels 10-30% above basal levels is required for wave emergence (67, 76, 77, 94-97). Anti-Mullerian Hormone (AMH), secreted by GC of growing follicles, is involved in the regulation of antral follicle

sensitivity to FSH during recruitment in humans (98). The antral follicle responds to increasing FSH concentrations by an increased rate of GC mitosis and by increased antral volume via aminoglycan production (77). The rise in FSH at the time of wave emergence induces expression of genes for FSH receptors (FSHr), the insulin-like growth factor (IGF) system, activin and the aromatase enzyme which regulate follicle growth beyond emergence (8, 77, 99-101). The number and health of the cohort of follicles that emerge in response to FSH is reflected in the rise in inhibin B (102). The GC of all antral follicles of the recruited cohort produce inhibin B. Inhibin B production peaks on the fifth day of the follicular phase (102). Research is required to further our understanding of the physiologic mechanisms underlying follicle wave emergence (1, 3).

2.2.2.1.2. Dominant Follicle Selection

Selection refers to the preferential growth of one follicle from a recruited cohort of antral follicles (3, 4, 7, 8, 20, 25, 74). Follicle selection always occurs during the ovulatory wave in the early to mid-follicular phase of the menstrual cycle (3, 7, 18). However, selection can occur following emergence in any follicular wave.

The selected follicle is called the "dominant follicle", while all other follicles of the wave are called "subordinate". At the time of selection, the growth profile of a selected follicle begins to diverge. Specifically, the dominant follicle continues to grow while the subordinate follicles enter a static phase or undergo atresia (7, 9). The divergence in growth trajectories occurs when the dominant follicle reaches a diameter of approximately 10 mm on day 6 to 9 of the follicular phase in women (3, 4, 7, 8). During the growth phase of the dominant follicle the emergence of the next follicular wave is suppressed (2, 9). It has been suggested that decreasing FSH in concert with increasing E_2 secretion are the primary

regulators of selection, regression and timing of emergence (2, 9). However, the complete mechanism of selection has not been fully elucidated (3, 77).

The interval of time that FSH is elevated above the threshold (rather than the magnitude of the rise in FSH) has been implicated in determining the number of follicles from the cohortselected for preferential growth (103, 104). The interval that FSH concentrations remain above the basal threshold levels is called the 'FSH window' (12, 103, 105-108). The duration of the FSH window aids in explaining the number of follicles that are selected to continue growing (12, 103, 105-108). In natural menstrual cycles, the duration of the FSH window is short such that typically only a single dominant follicle is selected. The decline in FSH occurring at the time of selection is critical the mechanism underlying the dominant between the growth of the dominant follicle and subordinate follicles (2, 9).

It has been postulated that the dominant follicle has an early size advantage (109, 110). The future dominant follicle may contain more GC and FSHr which increase the dominant follicle's sensitivity to FSH (103). At selection the declining FSH environment leads the subordinate follicles to undergo atresia (3, 4, 16). It is thought that the smallest follicles in the cohort are least sensitive to FSH and undergo atresia first, followed by the regression of progressively larger follicles in the wave until only the dominant follicle remains (9, 110).

Until follicle selection, pre-antral follicles of the recruited cohort continue to secrete AMH (111). Anti-Mullerian hormone production decreases in association with a rise in aromatase expression and E_2 synthesis in the GC (112). The expression of aromatase increases in 6-8 mm diameter follicles (4, 22, 25, 113-119). The dominant follicle produces more E_2 that the other follicles in the cohort prior to selection. The aromatase enzyme (discussed in section 2.3.2) is not fully functional until the follicular diameter exceeds 10 mm

(77). Production of androgens in the thecal cells by LH-induction provides the substrate for E_2 (12-14). The follicular fluid of the dominant follicle has a higher estrogen to androgen ratio, while atretic follicles exhibit a greater androgen to estrogen ratio (77, 118). The E_2 production of the dominant follicle contributes to the negative feedback on pituitary FSH secretion and the decrease in circulating FSH (120). Formation of LH receptors of the GC of the dominant follicle following E_2 secretion allows the dominant follicle to become more responsive to LH and less dependent on FSH following selection (15, 16, 121).

Inhibins A and B play critical roles in control of folliculogenesisPituitary secretion of FSH declines in concert with increasing inhibin B secretion in the early to mid-follicular phase (122-124). Increasing inhibin B is one component in inhibiting pituitary FSH secretion. Inhibin B production peaks at a follicle diameter of 9-10 mm (125) when inhibin A production increases (126). The production of inhibin A by the GC of the selected follicle increases androgen production by the thecal cells (127). An orderly transition from an inhibin B/activin environment to an inhibin A/follstatin environment has been proposed to be critical to the development of a dominant follicle in women (126, 128, 129).

The decreased dependency of the dominant follicle on FSH may also be the result of the acquisition of LHr by GC (15). Mural granulosa cells adjacent to the basal lamina, which are in close proximity to the theca interna vascular supply, express more LHr (93). Both FSHr and LHr are coupled to the cAMP signaling system. Thus, similar signal transduction responses occur with the binding of FSH and LH to their respective receptors (101). Serum LH concentration begins to increase in the mid-follicular phase (16, 130). In the presence of declining FSH and increasing LH concentrations, dominant follicle growth continues as a result of the additive effect of increased FSHr and LHr (131).

The dominant follicle is saved from atresia, in part, through increased IGF-I and II production and bioavailability (132, 133). The IGF system stimulates aromatase activity and estradiol production in the GC, while simultaneously promoting androgen production in thecal cells surrounding the growing dominant follicle (132, 133). The bioavailability of IGF-I and II is not increased in subordinate follicles. IGF is sequestered by IGF binding protein-4 (IGFBP-4) inhibiting GC and theca steroidogenesis and ensuring atresia (132, 134-136).

Continued growth of the dominant follicle is affected by a decrease in FSH and AMH, changes in the production of inhibin B to inhibin A, and an increase in aromatization of androgens to E_2 with the assistance of IGF-I and II. The follicle has developed a larger antrum and a large compliment of GC with an increased number of FSHr and LHr. The hormonal milieu allows the selected follicle to continue growing and attain pre-ovulatory status.

2.2.2.1.3. Pre-Ovulatory Follicle Growth

The process of dominant follicle maturation proceeds through the final two stages of follicle development, early pre-ovulatory and pre-ovulatory (Table 2.1)(77). The dominant follicle in an ovulatory wave increases from 10-24 mm on average, over the final 8 to 10 days of growth (66). The growth of the dominant follicle is associated with mitosis of GC and the accumulation of antral fluid (20). Growth occurs through proliferation of granulosa and thecal cells and the accumulation of antral fluid (20). By day 9 of a typical 16 day follicular phase, the density of the vasculature of the theca interna within the dominant follicle is twice that of all other follicles in the ovary; the vasculature facilitates preferential delivery of gonadotropins to the GC LHr (137).

The estrogen to androgen ratio increases as aromatase expression increases and estrogen production continues (77). Increased levels of inhibin A in CG stimulate thecal

androgen production. Elevated expression of IGF-II messenger ribonucleic acid (mRNA) in the GC stimulate aromatase enzyme activity. The venous effluent of the ovary containing the pre-ovulatory follicle has elevated levels of E_2. There is a correlation between increasing concentrations of serum E_2 and the size of the pre-ovulatory follicle (4, 138). The rate of granulosa cell mitosis declines as the GC and their nuclei increase in diameter at the end of pre-ovulatory follicle development (22). At this stage the basement membrane becomes less visible. The thecal layer continues to undergo hypertrophy and increased vascularization until ovulation occurs (66).

The dominant follicle is considered mature upon reaching pre-ovulatory size (16-28 mm). Ovulation occurs in response to a surge of LH at about day 14 to 16 of the follicular phase (77). A pre-ovulatory follicle will regress if a LH surge does not occur (77).

2.2.2.1.4. Ovulation

The elevation of E_2 in the late follicular phase triggers the pituitary release of LH (139). The rapid release of LH initiates the process of OV which involves complex biochemical, morphological, and physiologic changes in and around the 16-28 mm dominant follicle (3, 18, 22, 77, 140). The changes continue until the follicle wall ruptures and the oocyte is released. The pre-ovulatory follicle takes approximately 36 to 38 h to rupture/ovulate following the onset of the LH surge in humans (140). The biochemical and physiologic changes associated with OV are not fully elucidated (140).

The regulatory systems induced by the LH surge act synergistically to induce changes in the vasculature and extracellular matrix (140, 141). The extracellular matrix is stimulated to degrade by the LH surge. The LH surge stimulates the granulosa and theca intera cells to produce plasminogen activator (141-143). Plasminogen activator initiates the conversion of plasminogen to plasmin in follicular fluid. The plasmin generates collagenases which digest

the dense collagen layer of the tunica albuginea and the theca externa at the apex of the follicular wall (142, 144). Blood vessels of the theca interna invade the granulosa layer as the basal lamina breaks down immediately before OV (145).

Initiation of P_4 synthesis in the GC is induced by increased numbers of LHr and tonic LH secretion (146, 147). The LH surge suppresses GC mitosis and simultaneously stimulates formation of large lacunae within the GC. Changes in the GC following the LH surge results in a decrease in follicular fluid estrogen and androgen concentrations and increased production of P_4, prostaglandin E_2 (PGE_2; a vasodilator), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; a vasoconstrictor) before follicle rupture (146-149). However, the exact roles of P_4, PGE_2 and $PGF_{2\alpha}$ in follicle rupture remain poorly elucidated.

The pre-ovulatory LH surge also stimulates changes in the oocyte. The oocyte is stimulated to complete the first meiotic division and becomes arrested at prophase II as a secondary oocyte (20, 150). To stimulate the oocyte to complete the first meiotic division, the LH surge must reach a threshold concentration and be sustained for 14 to 27 h (141, 149, 150). The LH surge suppresses the action of oocyte maturation inhibitor (141, 149, 150). Final maturation of the oocyte and completion of meiosis will only be completed after fertilization (141, 149).

The final stages of the ovulatory process include expansion, loose reorganization, and increased vascularization of the follicular wall (151, 152). The follicle wall thins at the apex and thickens at the deep internal portion approximately 3 h prior to ovulation (151, 152). The apex is a small portion of the follicle wall digested by enzymes until the point where the tensile strength of the wall is no longer able to sustain the intra-follicular osmotic pressure (15-20 mm Hg)(148). A stigma forms at the follicular apex approximately 15 to 20 minutes (min) before follicle rupture due to thinning of the layers of collagen and

degradation of connective tissue elements (59, 152). The surface epithelium becomes necrotic and sloughs off at the apex of the follicle immediately before follicle rupture (153). The oocyte-cumulus complex, that is the oocyte surrounded by cumulus oophorus cells, floats freely in the follicular fluid just prior to OV. The follicular fluid and oocyte-cumulus complex evacuate the antral cavity when the stigma of the ovulatory follicle wall ruptures (144). Rupture of the follicle occurs at the stigma and within 15 seconds (sec) approximately 50% of the follicular fluid is released. The time required for complete evacuation of follicular fluid ranges from 6 sec to 18 min (152).

One of 3 structures are seen in the event of ovulatory failure: simple anovulatory follicular cysts, hemorrhagic anovulatory follicles (HAF), and luteinized unruptured follicles (LUF). Anovulatory follicular cysts may regress, remain static for several days and then regress, or grow past the typical pre-ovulatory diameter (> 25 mm) and persist prior to eventual regression (55, 151). Hemorrhagic anovulatory follicles are characterized by internal hemorrhage and fibrin network formation within the antrum (55). Luteinized unruptured follicles are characterized by a thickened (i.e., luteinized; discussed in section 2.2.2.2) follicular wall without release of the follicular fluid and the oocyte-cumulus complex. Luteinized unruptured follicles can hemorrhage internally. Regression of LUF occurs over a time period similar to that associated with normal CL regression (55).

2.2.2.2. Luteal Dynamics

The luteal phase begins immediately after follicle rupture with the formation of the CL. Luteinization is the process of CL formation through structural and functional remodeling of the theca and granulosa cell. The LHr on the cellular surface of transforming theca and granulosa cells allow for normal CL function(154, 155). Tonic secretion of low circulating levels of LH occurs during the early luteal phase.

Two parallel, but distinct events occur during initiation of luteinization. First, an extensive capillary network forms in the granulosa layer of the follicle following the disruption of the basement membrane (154). Second, the theca interna and mural granulosa cells transform into 2 populations of steroidogenically active lutein tissue (154). The former theca interna becomes small-luteal or paraluteal cells while the GC become large-luteal or luteal cells (154). Little is known about the mechanism of thecal cell remodeling; however, the transformation of GC into luteal tissue involves cellular hypertrophy with an increase in the nuclear to cytoplasmic volume ratio (155).

The two types of luteal cells differ in size, location, function, and regulation by endocrine and local factors (154). Paraluteal cells are less than 20 μm diameter and luteal cells are greater than 22 μm diameter (154). Paraluteal cells are located around the outer periphery and in the peripheral invaginations of the CL (154). Luteal cells are found in the central portion of CL tissue (154). Luteinizing theca and granulosa cells fill the antrum of the former follicle (154, 156). Some corpora lutea release their follicular fluid, then refill to form a 'cystic cavity' and do not completely involute (21, 156-159). Corpus hemorrhagicum (CH) results when a hemorrhage occurs within a developing luteal gland. A CH occurs in approximately 60% of ovulations (160). The structure of the CL is typically visible by 4 to 6 days following OV (154).

The paraluteal and luteal cells of the CL synthesize many hormones, including but not limited to P_4, E_2 and relaxin. Luteal cells secrete 10 to 20 times more P_4 than paraluteal cells (154). The principal endocrine change during luteinization is the production of P_4 (155). Luteinization increases the cellular receptors for low density lipoprotein (LDL). The receptors facilitate the intracellular transport of cholesterol for P_4 production (155).

The CL also contains several types of non-luteal cells. Endothelial cells are the most important type of non-luteal cell found. Endothelial cells are involved in luteal gland vascular development (154). The high number of endothelial cells found in the developing luteal gland along with the paraluteal and luteal cells correlate with increasing blood flow to the CL during the first 6 days of the luteal phase (161). The luteinizing follicle produces angiogenic factors, including vascular endothelial growth factor (VEGF) and vascular permeability factor (VPF). Angeogenic factors diffuse through the disrupted basement membrane and facilitate the formation of an extensive capillary network within the developing CL (155). Peak vascularity in the CL occurs 7 days after ovulation, which is the approximate day of maximum P_4 secretion (10-18 nanograms per millilitre; ng/mL) in a non-conception menstrual cycle (159, 162). The CL persists in the ovary in the presence of embryonic human chorionic gonadotropin (hCG) secretion which maintains P_4 secretion by the CL and prevents regression of the CL (163-166). Regression of the CL is referred to as luteolysis.

If pregnancy is not established, the CL begins to regress approximately 7 days after OV (165, 167). A decline in both the amplitude and frequency of LH pulses occurs in the mid-luteal phase. Declines in circulating estrogen and $PGF_{2\alpha}$, are involved in luteolysis. During luteolysis there is a decrease in the vascular supply and a decrease in cell size of the CL (167). Ultimately there is a loss in P_4 production (159).

Changes in the redox state of the luteal cells appears to regulate the lifespan of the CL (168, 169). The regulation of reactive oxygen species in the CL is managed in concert by $PGF_{2\alpha}$ and hCG (168-171). Human chorionic gonadotrophin directly suppresses the occurrence of apoptosis in the CL through prevention of oxidative stress while $PGF_{2\alpha}$ interaction with its receptor causes generation of free radical oxygen species and prolonged oxidative stress (168, 170). In domestic animals, $PGF_{2\alpha}$ is produced in the uterus; however,

in primates $PGF_{2\alpha}$ is produced by the CL (154). The regulation and action of $PGF_{2\alpha}$ in humans also may be influenced by its balance with PGE_2 which is a promoter of early luteal function (154).

The ratio of paraluteal and luteal cells changes as the lifespan of the CL progresses due to apoptosis and autophagocytosis (154). The alterations in CL cell ratios lead to gradual involution of the structure in the ovary (172). By the end of the menstrual cycle, involution of the luteal gland culminates in a small structure composed primarily of connective tissue called the corpus albicans (white body; CA)(172). The corpus albicans persists on the ovarian surface as a small white scar for several menstrual cycles (167).

2.3. Estradiol

2.3.1. Sex Steroid Hormone Pathway

The principal biosynthetic pathways for production of sex steroid hormones are summarized and are not unique to any specific tissue site (Figure 2.3). Sex steroid hormones are located at the end of the cholesterol steroidogenesis pathway. The major circulating form of steroidogenic cholesterol in humans is LDL (173). Delivery of LDL to the ovary allows for synthesis of androgens by thecal cells and aromatization of androgens to estrogen via the aromatase enzyme.

2.3.1.1. Estradiol Synthesis in the Ovary

The synthesis of E_2 in growing follicles occurs via interactions between GC and cells of the theca interna. Estradiol synthesis influenced by LH and FSH levels, the number of LHr and FSHr and the availability of precursor substrates. Studies characterizing E_2 synthesis in the ovary have been completed in the bovine model and are similar to human ovarian steroidogenesis (174, 175).

The vascular anatomy of the follicles in the ovary determine the degree to which circulating LDL serves as steroid precursors and the cells that are able to utilize LDL (173). Thecal cells of each follicle receive blood from the rich vascular supply of the ovary and are therefore in a position to utilize the circulating LDL. The basement membrane between the theca and granulosa layers of the follicle isolates the GC from direct contact with the ovarian blood supply (Figure 2.4)(173).

LH stimulates thecal cells to convert LDL to androgens (65, 77, 173). The availability of androgens produced by thecal cells controls E_2 secretion by GC (174). Androgens then cross the basement membrane into the GC where aromatase converts the androgens to estrogens (65, 77). Conversion of the androgens to estrogens involves a three-step reaction with aromatase in the granulosa cell mitochondrial agrandular endoplasmic reticulum (173, 176, 177). The final step in the production of estrogens is the aromatization of the androgen A ring (40, 178-181). The estrogens are subsequently released into the follicular fluid, intra-follicular space and systemic circulation (65, 77).

2.3.2. Aromatase Enzyme

The aromatase enzyme, or estrogen synthetase, is a microsomal member of the cytochrome P450 heme-protein containing enzyme complex superfamily (51, 181-184). Aromatase is responsible for catalyzing the conversion of androgens to estrogens is conserved across chordate species, sexes, and tissue types. In females, aromatase activity has been identified in the ovaries, placenta, brain, adipose tissue, muscle, liver, breast tissue, and malignant breast tumors (51, 180-185).

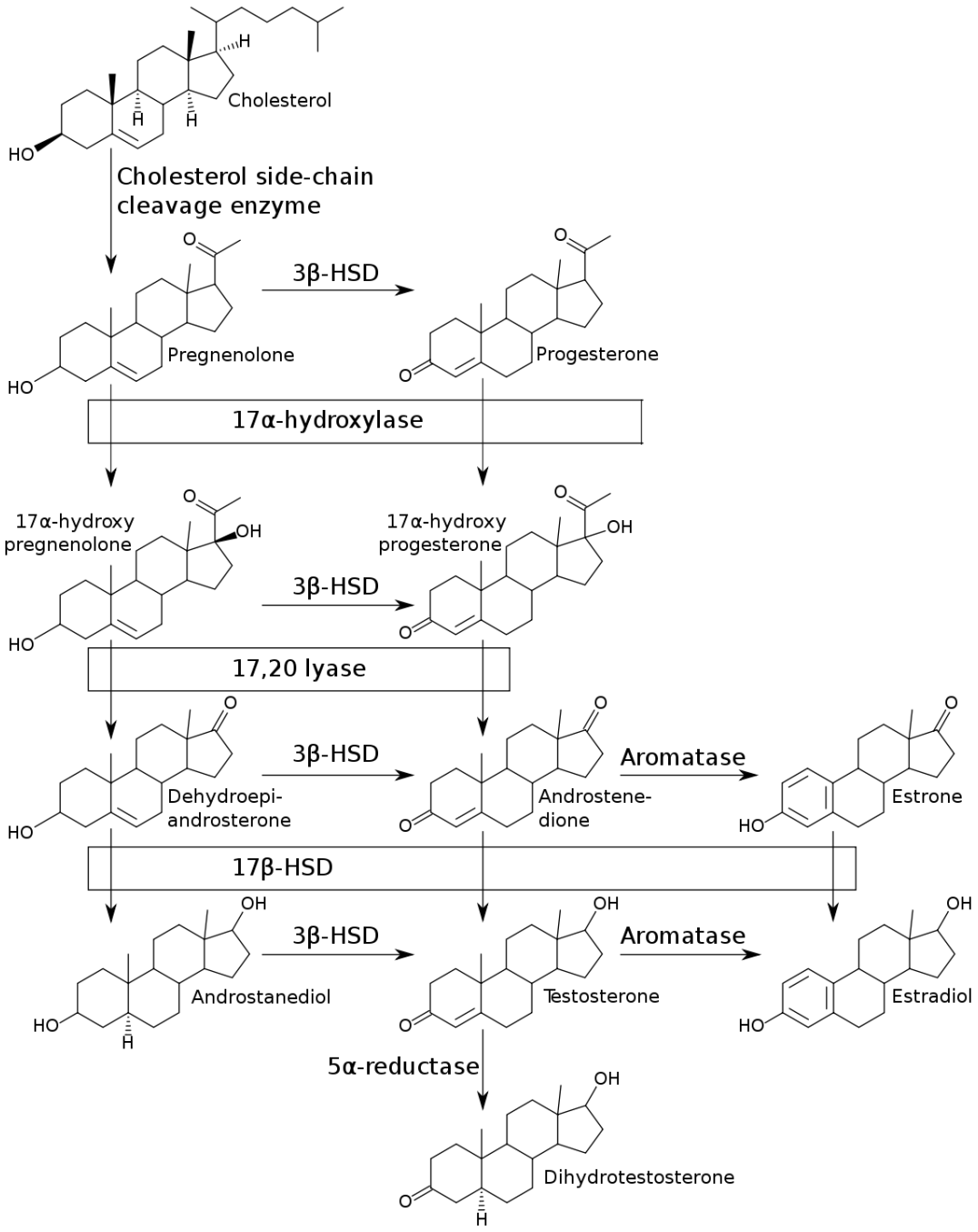

Figure 2.3: The principal biosynthetic pathways in the production of progestins, androgens, and estrogens from circulating vascular cholesterol. Cholesterol is metabolized into estrogens through 5 sequential enzyme systems. (Modified from Richfield, 2008).

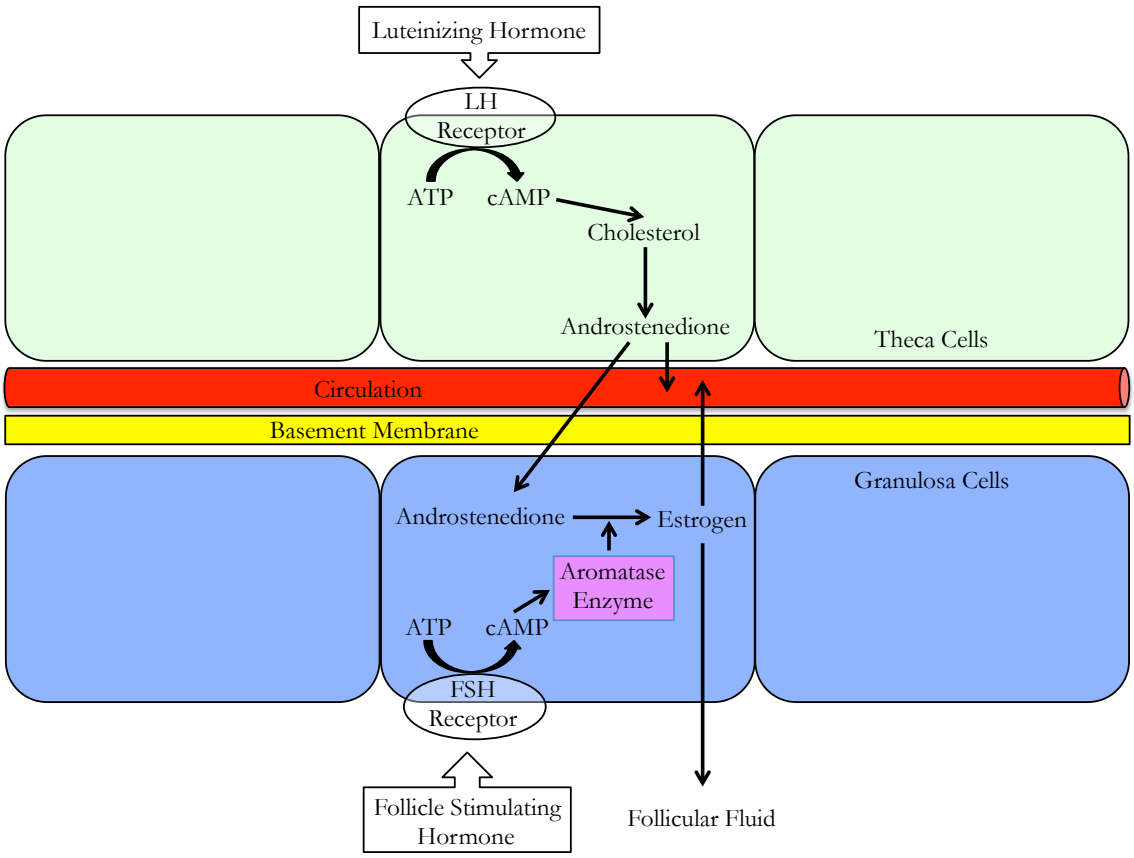

Figure 2.4: The 2-cell-2-gonadotrophin theory of estradiol production in the mammalian ovary (Modified from Adashi, Chapter 2, Reproductive Endocrinology, Surgery, and Technology, 1996)(65).

Transcription of aromatase in the GC of the ovary is induced by FSH (186). The aromatase enzyme is a 503 amino acid (aa) protein with a molecular weight of 55 kiloDalton (kDa)(178, 181, 187). The folding process produces 12 helices that are coded from A-L (188). A heme-containing protoporphyrin is located between helices I and L. The heme group is linked to the protein through a covalent bond with the sulfur atom of a cysteine aa and by a network of hydrogen bonds with positively charged aa (188). Substrate recognition occurs between helices B, C, F and G near the center of the folded enzyme (188). Interaction of substrates with the enzyme's active site occurs through a coordination bond with the heme group, hydrophobic bonding to approximately 10 aa and hydrogen bonding

to approximately 6 aa (188). Aromatase has the capacity to bind all 3 natural carbon 19 androgens (189) and catalyzes a 3 step reaction that rearranges the androgen A ring to an aromatic structure (173, 176, 177).

Aromatase binds to, and saturates, areas of the endoplasmic reticulum of cells in estrogen producing tissues tissues (40, 178-181, 189, 190). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) readily binds to membranes of the endoplasmic reticulum saturated with aromatase. Heterodimerization of aromatase with NADPH facilitates the conversion of androgens to estrogens (190). Following estrogen synthesis, nicotinamide adenine dinucleotide phosphate ($NADP^+$) is released and a new NADPH heterodimerizes with the aromatase enzyme.

2.4. Aromatase Inhibitors

2.4.1. Development of Aromatase Inhibitors

Aromatase inhibitors are compounds that act as false substrates for the aromatase enzyme through competitive binding with androgens for the heme moiety of aromatase (182-184). Aromatase inhibitors are classified as either steroidal or non-steroidal. Steroidal aromatase inhibitors are also named suicide inhibitors, type I inhibitors, mechanism-based inactivators, and aromatase inactivators (181-184, 191). Non-steroidal inhibitors are also called type II inhibitors and competitive inhibitors (181-183). Steroidal and non-steroidal inhibitor development started in the 1960s. Three developmental generations have occurred to improve the safety profile, increase potency and increase selectivity for aromatase binding (192).

2.4.1.1. Letrozole

Letrozole (Femara®, Novarts) is an achiral non-steroidal, third generation inhibitor of aromatase derived from triazole (Figure 2.5). Letrozole is completely absorbed after oral

administration and has a biological elimination half-life of 48 h (51, 182-184). An oral dose as low as 0.25 milligrams (mg) per day induced maximal plasma estrogen suppression in healthy women and breast cancer patients. A 98% decrease in circulating E_2 to 0.06 picomol per litre (pmol/L) was reported following letrozole administration (181). Side effects reported by trial subjects were rare but included gastrointestinal disturbances, asthenia, hot flashes, headache, and back pain (51, 183, 184).

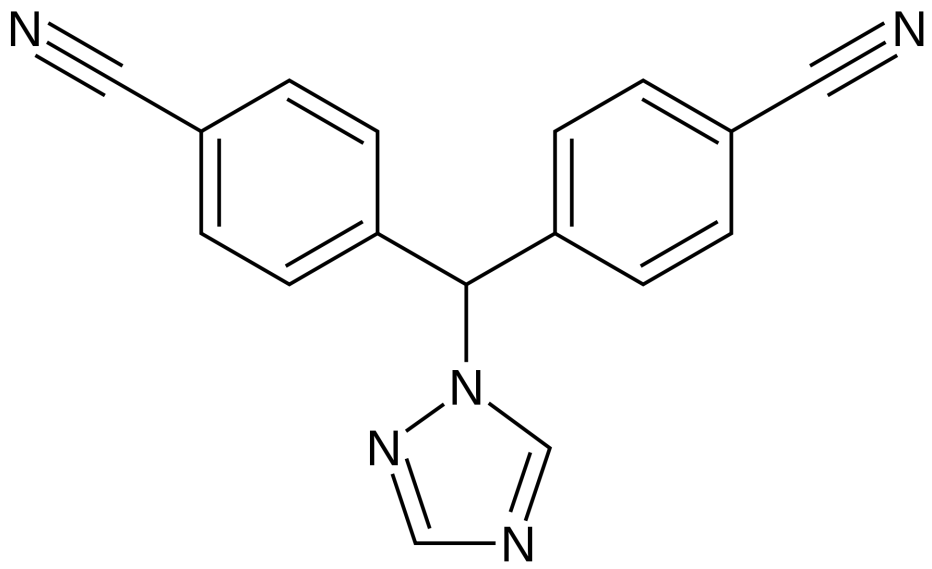

Figure 2.5: Chemical structure of letrozole. The base structure is a triazole group that competitively binds to the heme group of the aromatase enzyme. (from Lang, et. al, J Steroid Biochem Mol Biol, 1993)(193).

2.4.2. Use of Aromatase Inhibitors in Fertility Treatments

Commercially available aromatase inhibitors are registered solely for treatment of hormone-dependent breast cancer in post-menopausal women (194, 195). Letrozole is classified as embryotoxic and is contraindicated for pre-menopausal women who have ovaries *in situ* and any women attempting to become pregnant (194, 195). An increase in circulating concentrations of FSH and LH following letrozole treatment in mature female rats and bonnet monkeys was associated with the development of multiple normal pre-

ovulatory follicles (196). The rationale for AI use as a fertility therapy in humans was to remove circulating E_2 and release the hypothalamic/pituitary axis from the negative feedback of E_2 (182, 197). The release of negative feedback results in an increase in FSH secretion and stimulation of ovarian follicle growth (44, 45, 49-51). There are numerous reports of aromatase inhibitor use in the treatment of infertility, for women undergoing ovarian stimulation, intrauterine insemination and timed intercourse (106, 198-205). Aromatase inhibitors have been used before follicle selection for ovarian stimulation as a large single dose or low dose 5 day regimen (43, 48-50, 182, 196, 200-202, 206-211). Aromatase inhibitors also have been used in combination with FSH to decrease the dose of gonadotrophins required to achieve an acceptable stimulatory response (45, 47, 48, 199, 205, 212-216). Use of AI in fertility therapy has been successful in achieving pregnancy; however, concerns still remain regarding fetal safety following AI use.

A retrospective study presented in 2005 by Biljan challenged the safety of letrozole use in infertility treatment. Increased risks of bone malformations and cardiac anomalies were correlated with the use of letrozole in infertility treatment (217). However, the increased risks of congenital anomalies following letrozole use (previously reported) were not higher than the reported 1.3 fold increased risk of congenital anomalies for babies born after all assisted reproductive therapies above the baseline rate of anomalies (2-4%) without treatment (218, 219). Limitations in study design have led to questions regarding the scientific credibility of the Biljan study. Teratogenic effects on fetal development were not observed in a more recent retrospective study on the use of letrozole in fertility therapies (220). A prospective trial to evaluate pregnancy outcomes after ovarian stimulation with AI further confirmed the safety of AI as a tool for management of infertility in women (221).

2.5. Emergency Contraception

Emergency, or post-coital, contraception is often called "the morning-after pill". Emergency contraception is intended to prevent pregnancy when used 72 to 120 h after unprotected intercourse or following failure of other forms of contraception (222-226). Emergency contraception is rooted in 1920s veterinary medicine practice where estrogenic ovarian extracts were injected intramuscularly to prevent conception following unintentional breedings (31, 32). The first published cases of estrogen injections for EC in humans were described in the mid-1960s (31, 32). In an effort to increase the efficacy of pregnancy prevention, the use of combined estrogen/progestin and progestin only hormonal combinations as EC was investigated in the 1970s.

Two hormonal EC regimens and one non-hormonal EC method are currently available in Canada (227). Plan B™ is a hormonal emergency contraceptive consisting of 2 oral doses of 0.75 mg levonorgestrel (LNG) taken simultaneously (227, 228). The second hormonal method is the Yuzpe method, off label use of oral contraception. Treatment with the Yuzpe method consists of 2 doses of a combination of 0.1 mg ethinyl estradiol (EE) and 0.5 mg LNG taken 12 h apart (227, 229-231). The non-hormonal method includes the insertion of a copper intrauterine contraceptive device (IUCD) prior to the time an embryo would be transported from the site of conception in the oviduct to the uterine cavity. A new hormonal method, ulipristal acetate, was approved for use in August 2010 by the United States Federal Drug Administration (FDA)(232). Ulipristal acetate, Ella™, is a selective progesterone-receptor modulator and is designed to be taken as a single 30 mg oral tablet within 120 h of unprotected intercourse or barrier contraceptive failure (232).

The mechanisms of action of hormonal emergency contraceptives remain poorly elucidated. The two main hypotheses to explain their contraceptive action include

prevention of fertilization and/or prevent implantation. Prevention of fertilization may occur by preventing follicular development, and/or ovulation or by affecting sperm function and/or sperm migration through the female reproductive tract (30, 233-236). Prevention of implantation is thought to involve an alteration in endometrial receptivity and/or luteal function (30, 234-236).

2.5.1. Ovarian and Endometrial Function following use of EC

Follicular development, ovulation, and gonadotropin response to the administration of EC have been studied (28-30, 32, 229, 235-246). Emergency contraceptives are effective at preventing pregnancy when administered at follicle diameters < 12 mm (35, 236). Pregnancy is less likely to occur following unprotected intercourse at this time regardless of whether or not emergency contraceptives are administered because OV is unlikely to be imminent (35, 236). A delay or blunting of a LH surge has been observed following EC administration in the presence of a dominant follicle < 17 mm in diameter (28, 30, 35, 234, 246); no effects were observed at a follicle diameter of 18 mm (28, 30, 35, 234, 236, 246). Hormonal EC also have the potential to interfere with the formation or endocrine function of the CL. This mechanism of action is dependent upon the time of the LH peak and EC regimen used (242, 243).

Endometrial receptivity following EC use has also been studied. Use of LNG contraceptives before a LH surge did not affect the morphology of the endometrium or any known markers of receptivity at the expected time of implantation (243, 247, 248). In contrast, EC use in the early luteal phase has been shown to cause alterations to endometrial function at the cellular level (249-251). Changes to the normal IMI of participants were observed after a single treatment with LNG (252). The change in IMI length was dependent on the timing of EC initiation (252).

2.6. Ultrasonographic Imaging in Female Reproductive Physiology

2.6.1. Overview of Ultrasound Imaging

The study of ovarian follicular development was revolutionized by the development of non-invasive ultrasonographic imaging. Ultrasonography provided an atraumatic and non-invasive tool with which to observe serial changes in reproductive function not possible with endocrinologic or histologic evaluation (151, 152). Serial ultrasonography can be used to increase our understanding of reproductive physiology, pathophysiology and improve the clinical diagnosis of ovarian and uterine disease (55, 253).

Medical diagnostic ultrasonography uses high frequency sound waves (above 20 kHz) to generate images of stationary and moving tissues in the body (253-256). Briefly, ultrasound systems are composed of a transducer, computer processor, and monitor. The transducer contains piezoelectric crystals which expand and contract to emit sound waves. Tissues in the body either reflect or propagate the acoustic pressure waves depending on the acoustic impedance of the tissue. Changes in tissue density at tissue interfaces determine the echotexture (proportion of the acoustic pressure wave reflected, absorbed or scattered) of the tissue which in turn determines the amplitude of the returning ultrasound wave. Dense structures reflect most of the acoustic pressure waves and appear hyper-echoic, while fluid-filled structures propagate the waves and appear hypoechoic (52). The reflected acoustic pressure waves cause the piezoelectric crystal in the transducer to compress and subsequently generate an electrical signal. The electrical signals are amplified and then stored as binary digits in a digital scan converter. The binary information is stored in a matrix of picture elements (pixel) which are then displayed on a monitor.

Each pixel represents a discrete tissue reflector. The go-return time of the echo determines the depth of the tissue reflecting the sound wave. The deeper the tissue, the

weaker the amplitude of the returning echo. The brightness of a pixel is related to the amplitude of the reflected signal and is displayed as one of 256 shades of grey (hypoechoic, black = 0 and hyper-echoic, white = 255)(255). Balancing the brightness of the echoes that originate from distal reflectors and proximal reflectors in the displayed image is done through application of internal gain controls. Numerous acoustic pressure beams are focused to increase lateral resolution, while axial resolution is optimized through increased ultrasound frequency (256).

Received echoes can be displayed in different modes. Amplitude mode (A-mode) is useful for accurate transducer-to-tissue distance measurements by mapping the signal amplitude versus reflection distance of the returning echo signal (253, 256). Brightness mode (B-mode) displays the received echo signals as intensity modulated dots on the screen (253, 256). Motion mode (M-mode) is the sweeping of the B-mode trace across the screen while holding the ultrasound beam stationary. M-mode displays motion of tissues in the track of the sound waves (253, 256).

In research and clinical examinations of the female pelvis, transvaginal ultrasonography (TVS) is preferred over transabdominal ultrasonography due to the increased resolution (257, 258). The ovaries and uterus are easily visualized using B-mode TVS due to the close proximity of the organs to the transducer. Transvaginal ultrasonography may be utilized to monitor ovarian and endometrial changes during natural menstrual cycles, infertility treatments, post menopause, hormonal contraceptive use, and research (52). Clinicians also use TVS to aid in invasive procedures, detect and monitor early pregnancy and assess reproductive tissues for anomalies (55, 259). Additionally, TVS is used for elucidating the effects of treatments on ovarian and uterine function.

2.6.2. Ultrasonographic Image Characteristics of the Ovary

Ovarian tissue images are characterized by a coarse low-level echo pattern (52). Interspersed within the low level echoes comprising the ovarian stroma are hypo-echoic spheres which represent ovarian follicles (52). Follicles as small as 2 mm in diameter can be detected (Figure 2.6). The detection of dominant follicles occurs simultaneous to a rise in plasma E_2 (4). Mature pre-ovulatory follicles measure 18-24 mm in diameter (52). Changes in pre-ovulatory follicle morphology associated with impending OV (discussed in 2.2.1.4) appear as decreased echogenicity of the follicular wall (52, 151). Following OV, the developing CL can be observed at the location of the former pre-ovulatory follicle (described in 2.2.2; Figure 2.6)(152, 167, 260). The point of follicular rupture can be observed with ultrasonography for up to a week following OV (55). Ultrasonographic characteristics of the CL are highly variable due to the macroscopic changes that occur during luteal formation. The outer surface of the CL has a hyperechoic echotexture compared to the relative hypoechoic echotexture of the central portion of cystic CL (167).

The ultrasonographic echotextural characteristics of the 3 types of ovulatory failure (discussed in 2.2.1.4.1) can be used to assess if anovulation has occurred. Anovulatory follicles have predominantly thin, hyper-echoic, clearly demarcated follicular walls of uniform thickness that is characteristic of a cohesive tissue layer (151). Hyperechoic free-floating and protruding structures in the antral cavity are commonly observed (55). Luteinized unruptured follicles have thick follicular walls with an echotexture similar to luteinized tissue, an indistinct antral border and no point of follicle rupture (55).

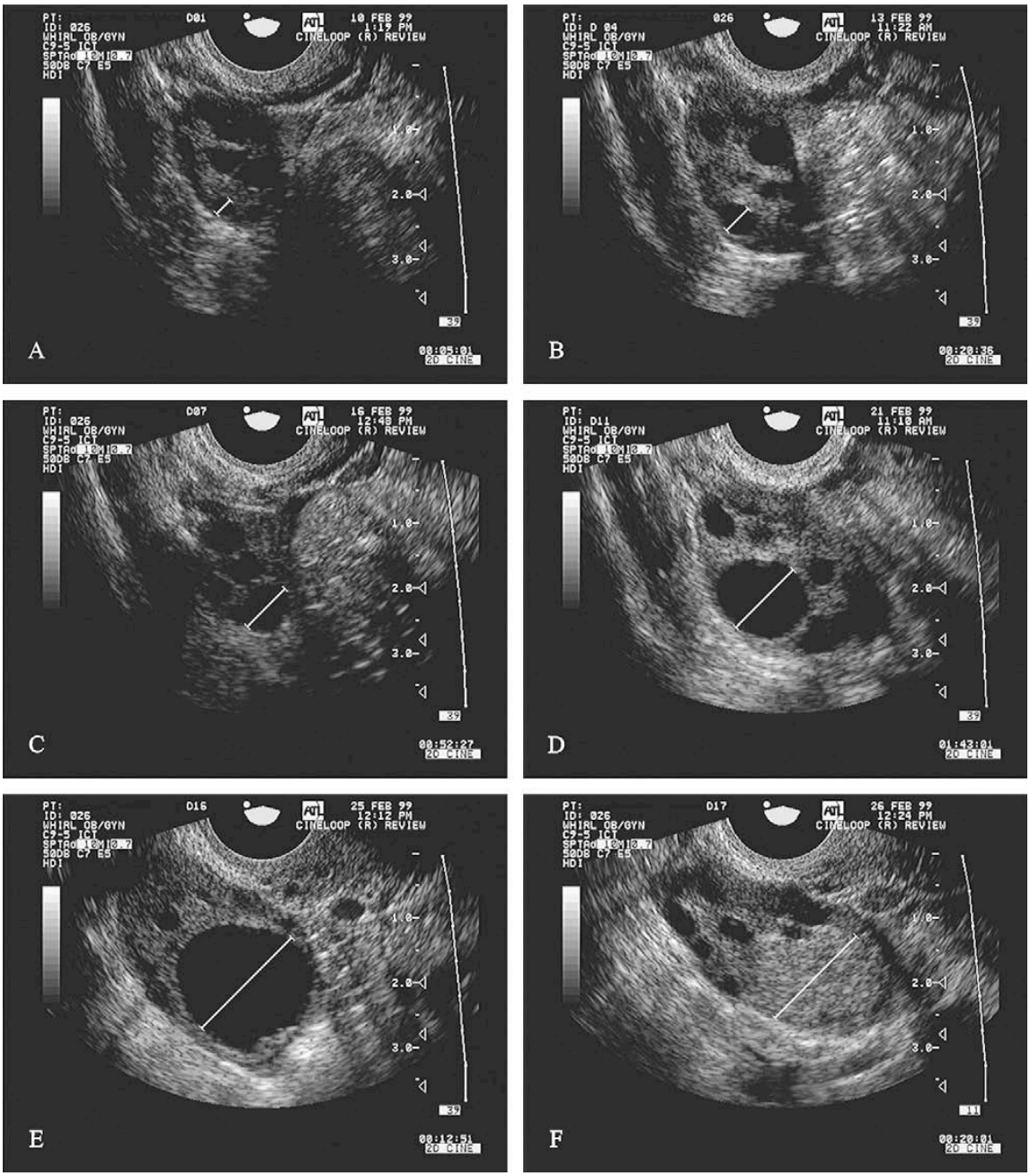

Figure 2.6 a-f: Serial transvaginal ultrasonographic images of the right ovary of a research participant on days 1 (A), 4 (B), 7 (C), 11 (D), 16 (E), and 17 (F) of a spontaneous menstrual cycle demonstrating different stages of follicle and a luteal dynamics. The same ovarian follicle is identified throughout the growth phase (A-E) and the corresponding CL on the day following ovulation is shown (F) (from Baerwald, et. al, Fertility and Sterility, 2009)(261).

2.6.3. Ultrasonographic Image Characteristics of the Human Uterus

The myometrium is displayed with homogenous echotexture and echoes of medium intensity (52, 55). The echotexture of the myometrium does not change during the menstrual cycle (55). The lumen of the uterus appears as a hyperechoic line from the fundus to the cervix in the sagittal plane. In the transverse plane, the uterine lumen appears as a horizontal hyperechoic line with the opposing layers of the endometrium appearing as an echoic oval surrounding the lumen (52).

The echotexture of the endometrium varies throughout the menstrual cycle in concert with circulating concentrations of E_2 and P_4 (Figure 2.7)(55). An A pattern describes the echotexture immediately post menses and is observed as a hyper-echogenic thin, single line with no detectable differentiation of the stratum functionalis and basalis (262). During the early follicular phase, a faint triple line echotexture pattern is observed reflecting the separation of the stratum functionalis and basalis layers and is termed a B pattern (262). A C pattern is observed during the peri-ovulatory period of the follicular phase. It is characterized by a thick, pronounced triple line of hypo-echoic, hyper-echoic, and hypo-echoic layers (262) which represents pronounced differentiation of the stratum functionalis and basalis (262). A thick, homogeneous, moderate echotexture is the endometrial pattern typically observed during the luteal phase and is termed a D pattern (262). An M pattern is indicated when active menstrual flow is observed during menses and stratum functionalis and basalis appear as a hyperechoic, thin tissue layer (262).

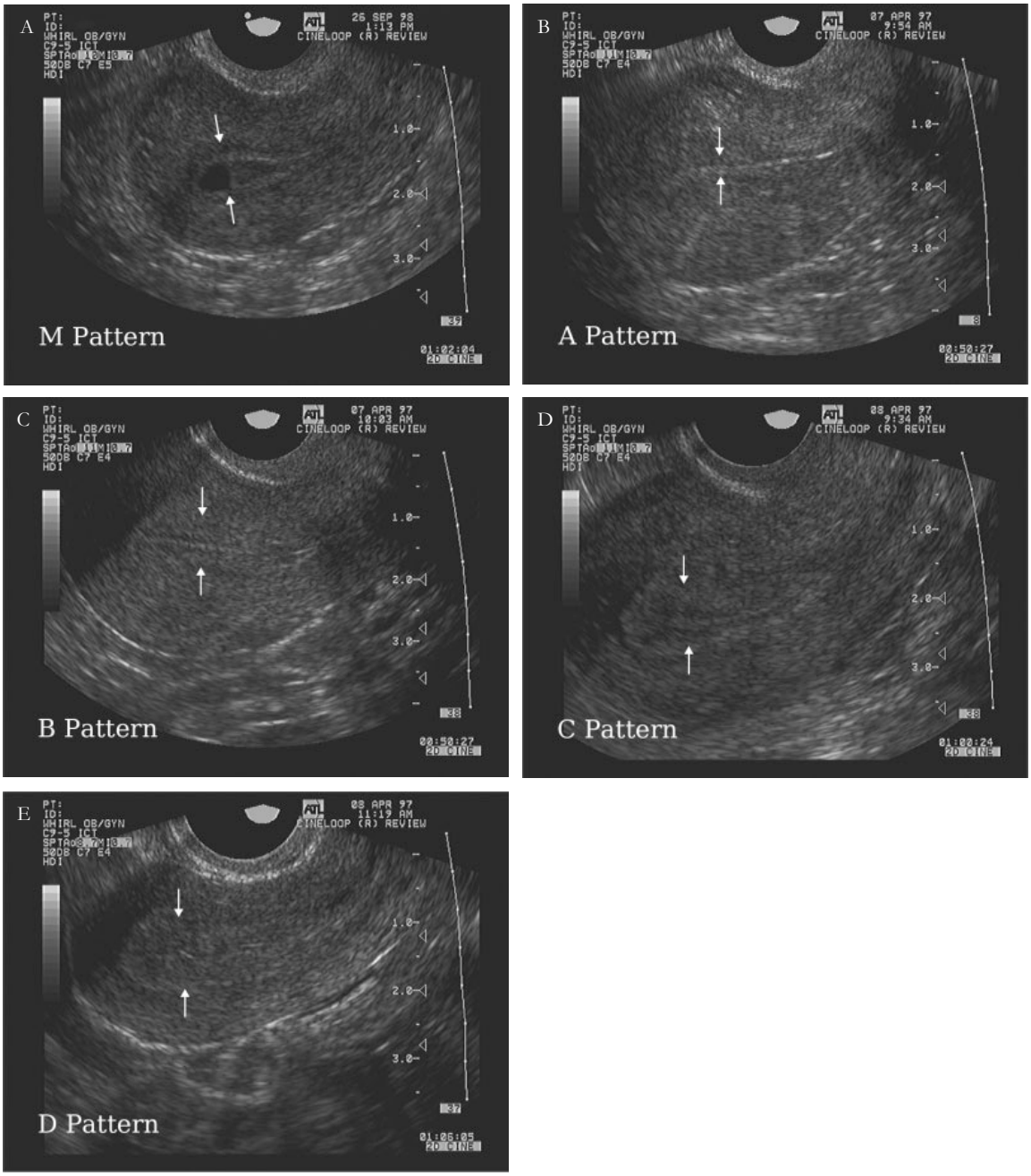

Figure 2.7 a-e: Transvaginal ultrasonography of the endometrium in the sagittal plane with arrows identifying the anterior and posterior borders of the endometrium. The fundus of the uterus is to the left of the images and the cervix is to the top right of the images. The images illustrate the echotexture of an M pattern (A; day 3 of menses, active flow visualized), an A pattern (B; early follicular phase), a B pattern (C; mid-follicular phase), a C pattern (D; peri-ovulatory period), a D pattern (E; mid-luteal phase)(from Baerwald, et. al, Ultrasound in Obstetrics and Gynecology, 2004)(262).

Chapter 3: Rationale

Human ovarian folliculogenesis occurs in a wave-like pattern throughout the IOI. Two or three waves (or cohorts) of follicles develop through out an IOI. The final follicle wave in an IOI is ovulatory; an observation that is consistent across species. Recruitment of a cohort of follicles into a developing follicle wave is dependent upon a rise in FSH above a threshold level. In women, the pre-ovulatory follicle is selected for preferential growth over other follicles in the wave at approximately 10 mm in diameter. At this time, the aromatase enzyme complex in the dominant follicle becomes fully functional and the selected follicle produces increasing amounts of E_2 (77). The selected follicle's production of E_2 induces the formation of LHr on the GC which then primes the dominant follicle for the mid-follicular phase surge in LH and subsequent OV.

Emergency contraception was originally developed with the intent of suppressing follicular development, OV, or the ability of an embryo to implant in the uterus (28-30). However, the response of the ovaries to current hormonal EC is unpredictable. Exogenous E_2 and P_4 are effective at preventing pregnancy when administered at follicle diameters < 12 mm (35, 236) although pregnancy is less likely to occur following unprotected intercourse at this time regardless of whether or not emergency contraceptives are administered. Emergency contraception does not inhibit OV when administered in the presence of a pre-ovulatory dominant follicle ≥ 18 mm (33-36). Ancillary support for this reasoning is indicated by reports from Canada and the United States indicating approximately half of annual pregnancies (450,000 and 3.5 million, Canada and US respectively) are unintended and half of unintended pregnancies end in elective termination (263, 264). The inability of EC to prevent OV is problematic given the routine clinical use of the medication.

Aromatase inhibitors prevent the enzymatic conversion of androgens to estrogens and subsequently lower the concentration of serum estrogen (37-41). When AI are administered before selection as part of fertility treatments, pre-ovulatory sized follicles are typically observed 10 to 12 days later (42-49). The inhibition of aromatase prevents E_2 production and releases the hypothalamic/pituitary axis from the negative feedback of E_2. The release results in an increase in FSH secretion and stimulation of further growth of the extant wave (44, 45, 49-51). Administration of AI in the presence of a selected follicle has not been documented.

The rationale for the trial comprising the work in this thesis was developed from the idea that a non-steroidal AI would cause an acute drop in E_2 at physiologically critical time points of the natural menstrual cycle based on observations from infertility treatments and EC. The dose selected was based on reports of multiple and single dose regimens in fertility therapy and is slightly less than half the maximal multiple dose regimen. We theorized that if AI were administered after follicle selection the acute E_2 deprivation would initiate atresia of the dominant follicle in the extant wave, as is observed in defined time OC administration. Estradiol deprivation would be expected to release negative feedback on FSH and result in the emergence of a new follicular wave without an ovulatory event occurring.

We anticipate that our study will increase our understanding of the role of E_2 in follicle selection, OV induction, and early luteal formation. A greater understanding the control of human ovarian dominant follicle selection, OV induction, and early CL development has implications in the development of emergency contraceptives and fertility treatments. Information regarding the mechanisms of follicular wave emergence, dominant follicle selection, and CL formation could be used to develop novel ways to use current

hormonal contraceptives, develop new contraceptive technologies, and provide more contraceptive choices for women.

Chapter 4: General Objectives and Hypotheses

The general objectives of the study contained in this thesis were to:

1. characterize ovarian follicular and luteal development following AI administration post selection in the follicular phase;
2. characterize the endometrial response to AI administration post selection in the follicular phase; and,
3. assess the response of FSH, LH, E_2, and P_4 to AI administration post selection in the follicular phase.

We hypothesized that a single 20 mg dose of AI administered at peri-selection diameter, pre-ovulatory diameter, or 24-48 hours post-OV in the natural menstrual cycle would cause ovulatory failure and/or failure of CL formation which would be associated with arrested endometrial development, a shortened interval to menses, and new follicular wave emergence. We hypothesized that a single 20 mg dose of AI at these times would cause an acute drop in E_2 which would be associated with a rise in FSH and no change in LH.

Chapter 5: A single 20mg dose of aromatase inhibitor does not inhibit folliculogenesis or luteogenesis

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Abstract

Introduction: Ovarian folliculogenesis occurs in a wave-like pattern of growth during the menstrual cycle. A better understanding of the role of estrogen in folliculogenesis may lead to the development of better contraceptive and fertility protocols. Aromatase inhibitors cause a transient decline in natural estrogen production. Multiple small doses of an aromatase inhibitor have been used during fertility therapy to induce dominant follicle growth and ovulation.

Objectives: Our objectives were to elucidate the effects of a single 20 mg dose of an aromatase inhibitor (Letrozole/Femara™) on folliculogenesis and test the hypothesis that an AI administered during defined times of the follicular phase or immediately after ovulation would result in atresia of the extant dominant follicle and initiate new wave emergence.

Methods: Healthy, reproductive age, female volunteers not taking hormonal contraceptives were recruited (n=41). Ultrasound examinations began on day 4 of the menstrual cycle (day 1=first day of menses). Volunteers were randomized into 1 of 4 groups: treatment at a follicle diameter of 1) 12 mm, 2) 18 mm, 3) the first day following ovulation, or 4) control. Serial ultrasonography and phlebotomy were performed until ovulation in the subsequent

cycle. Differences were analyzed using t-test, ANOVA, repeated measures ANOVA, or Kruskal-Wallis ANOVA where appropriate.

Results: The dominant follicle in all treatment groups ovulated. There were no differences among experimental groups for peak follicle diameter, follicular growth rate, endometrial thickness at ovulation, or inter-ovulatory interval. Maximum FSH concentrations in the 12 mm, 18 mm and post-OV groups were 12.58 ± 1.41 mIU/mL, 18.62 ± 2.27 mIU/mL, 12.38 ± 1.10 mIU/mL, respectively. Maximum LH concentrations in the 12 mm, 18 mm and post-OV groups were 16.20 ±2.06 mIU/mL, 40.43 ± 4.32 mIU/mL, 16.34 ± 2.59 mIU/mL, respectively. The 18 mm group had higher FSH and LH concentrations ($P < 0.02$) compared to the 12 mm and post-OV groups.

Conclusions: Administration of a single 20 mg dose of an aromatase inhibitor at defined times of the menstrual cycle did not induce dominant follicle regression or new wave emergence. Treatment resulted in continued follicle development, a transient decrease in E_2 levels and elevated circulating FSH and LH concentrations. The failure of an aromatase inhibitor to interrupt dominant follicle development suggests a compensatory mechanism for the acute drop in E_2 that may involve increased LH and FSH levels.

Introduction

The suppression of follicular development, ovulation (OV), or the ability of an embryo to implant is the intent of emergency contraception (EC) in clinical practice (29, 30). Emergency contraceptive regimens use various formulations of exogenous estrogens and progestins to prevent pregnancy and are now available 'over-the-counter' in many countries around the world, including Canada (265, 266). The use of EC by women is increasing as women become aware of the availability of post-coital means of preventing pregnancy (267, 268).

The ovarian response to EC regimens is unpredictable and OV is not inhibited when EC are administered near the time of natural OV (28, 34-36). Ancillary support for the need of more effective EC is indicated in data from Statistics Canada (269, 270). In Canada approximately 450,000 pregnancies are documented annually - approximately 120,000 end in elective termination (269, 270). In the United States there is a similar trend, 3.5 million pregnancies occur annually, 1.72 million are unintended and nearly 1 million unintended pregnancies end in elective termination (263, 264).

Women are educated about their menstrual cycles based on the traditional theory of follicle development. The traditional theory states that the human menstrual cycle is visualized from the first day of menses to the day before the next first day of menses (20, 66). Menstruation is the external sign of reproductive cyclicity in women. The traditional theory also states that a single cohort of antral follicles is recruited for growth in the late luteal phase (20, 66). Transvaginal ultrasonography has facilitated the use of OV as the definitive endpoint of the reproductive cycle. Follicle growth is thus observed from OV to OV, which is termed the "inter-ovulatory interval" (IOI). Ultrasonography has also facilitated the identification and characterization of wave-like patterns of antral follicle growth during the human menstrual cycle (3, 18). Documentation of follicular waves in women is consistent with follicular wave dynamics of the estrus cycle in several domestic animal species (78, 84, 90, 92, 271). When the menstrual cycle in women is observed over an IOI menses occurs in the middle of the IOI and the final wave that occurs is ovulatory.

Current formulations and administration regimens of hormonal EC are based on the traditional theory of follicle development. These methods are intended for use up to 120 hours after contraception failure or unprotected intercourse (222, 223, 227, 272, 273). The two standard methods available in Canada are Plan B^{TM}, 0.75 mg of levonorgestrel, and the

Yuzpe method, 0.1 mg ethinyl estradiol and 0.5 mg levonorgestrel (28, 35, 227, 272). Both methods require two doses to be taken 12 hours apart. Ovarian follicular development and OV in women have been studied after use of both regimens; however, the mechanisms underlying follicular growth, regression and OV following EC use remain poorly elucidated (28, 35, 36, 274). The mechanism of action was different with each regimen and depended upon the relative timing between intercourse, administration, and expected OV (28, 35). The two main hypotheses to explain the contraceptive action of EC are the prevention of fertilization and implantation (30, 233-236). Hormonal EC is most effective if administered when the dominant follicle diameter is small (i.e., 4 to 5 days prior to expected OV); however, during this time interval intercourse is less likely to result in pregnancy whether or not EC is administered (28, 34-36).

Aromatase inhibitors (AI) prevent the enzymatic conversion of androgens to estrogens (41, 50, 51). Letrozole (Femara™) is a non-steroidal aromatase inhibitor that reversibly binds to and inactivates the aromatase enzyme (48, 182, 183). Letrozole was designed as an adjuvant therapy in estrogen positive post-menopausal breast cancer (275, 276). Letrozole also has been used for the treatment of infertility (50, 203). Administration of AI before follicle selection was proposed to temporarily release the hypothalamic-pituitary axis from estrogenic negative feedback (44, 46, 49-51). The feedback release would result in increased FSH secretion and stimulate further growth of the extant wave. Growth of a dominant follicle would increase E_2 secretion and induce normal negative feedback at the hypothalamic-pituitary axis (44, 46, 49-51). Following this proposed mechanism of action, small 5-day dose and large single dose regimens of letrozole have been used successfully to induce follicle growth (45, 182, 206). The small sample sizes in studies utilizing letrozole prevent conclusions about the efficacy of AI in OV induction from being complete.

Recently, the effects of a single or 3 day intravenous dose of letrozole on folliculogenesis in beef heifers has been evaluated. Letrozole therapy did not induce follicular atresia, hasten new follicular wave emergence, or induce increased FSH concentrations when administered in anovulatory or ovulatory waves (277, 278). Letrozole administration in heifers increased plasma LH concentrations and extended the period of follicle dominance that led to delayed emergence of the next follicular wave (277).

Based on evidence from EC reports we proposed that after follicle selection antral follicles would respond differently to AI treatment compared with known pre-selection responses to AI in fertility therapy. The overall objectives of the present study were to: 1) characterize ovarian follicular, luteal and endometrial development following a single 20 mg dose of an AI (Letrozole/Femara^{TM}) using high-resolution transvaginal ultrasonography; and, 2) to assess the response of FSH, LH, and E_2 to a single 20 mg dose of letrozole. We hypothesized that a single 20 mg dose of letrozole administered at defined points of follicle development in the natural menstrual cycle would cause ovulatory failure and/or failure of CL formation and be associated with arrested endometrial development, a shortened interval to menses, and new follicular wave emergence. We also hypothesized that a single 20 mg dose of letrozole at defined times of follicle development would cause a transient drop in E_2 which would be associated with a rise in FSH and no change in LH. The defined points of follicle development were chosen to represent peri-selection (12 mm), pre-ovulatory (18 mm), and post-ovulatory (24 hours post-OV).

Materials and Methods

A prospective, randomized, controlled, single centre, open label trial was conducted. The study protocol was approved by the Biomedical Research Ethics Review Board at the University of Saskatchewan and Health Canada. All study procedures were conducted in full

compliance with the Tri-Council Policy Statement on the Ethical Conduct for Research Involving Humans, ICH Good Clinical Practice Guidelines and Declaration of Helsinki at the Women's Health Imaging Research Laboratory at the University of Saskatchewan.

Participants

Forty-one women between the ages of 18 and 35 years (25.48 years ± 0.86) with a body mass index (BMI) of 18 to 32 (25.04 ± 0.55) were enrolled. Informed consent was obtained from all women prior to the initiation of study procedures. All participants were non-smoking, healthy women with regular menstrual cycles and no contraindication to contraceptive or AI use (as determined following medical history and physical examination). The women were required to have discontinued use of hormonal contraceptives 2 or more months prior to beginning the study. Participants were asked to abstain from intercourse during the study; however, participants were also provided with barrier contraceptives (condoms) throughout the study to prevent pregnancy.

Treatments

Women were randomly assigned to 1 of 4 experimental groups at the first ultrasound: AI administration 1) at a first detection of a 12 mm ± 1 mm follicle diameter in the follicular phase (n = 10), 2) at an 18 mm ± 1 mm follicle diameter in the follicular phase (n = 10), 3) within 24 hours of OV (n = 10), and 4) a contemporaneous no treatment control group (n = 11). The randomization procedure was a randomized complete block design. All women in the treatment groups received a single 20 mg dose of letrozole (Femara™, Novartis Pharmaceuticals Canada Inc, Dorval, Quebec) when they reached the follicle size at which they were randomized to receive treatment. Participants were followed for 1.5 menstrual cycles (one inter-menstrual interval, followed by the interval from menses to OV of the next cycle). The experimental period was defined as 5 sequential days

beginning on the day of treatment. A schematic representation of the study protocol, treatment initiation, ultrasound, and phlebotomy is shown (Figure 5.1).

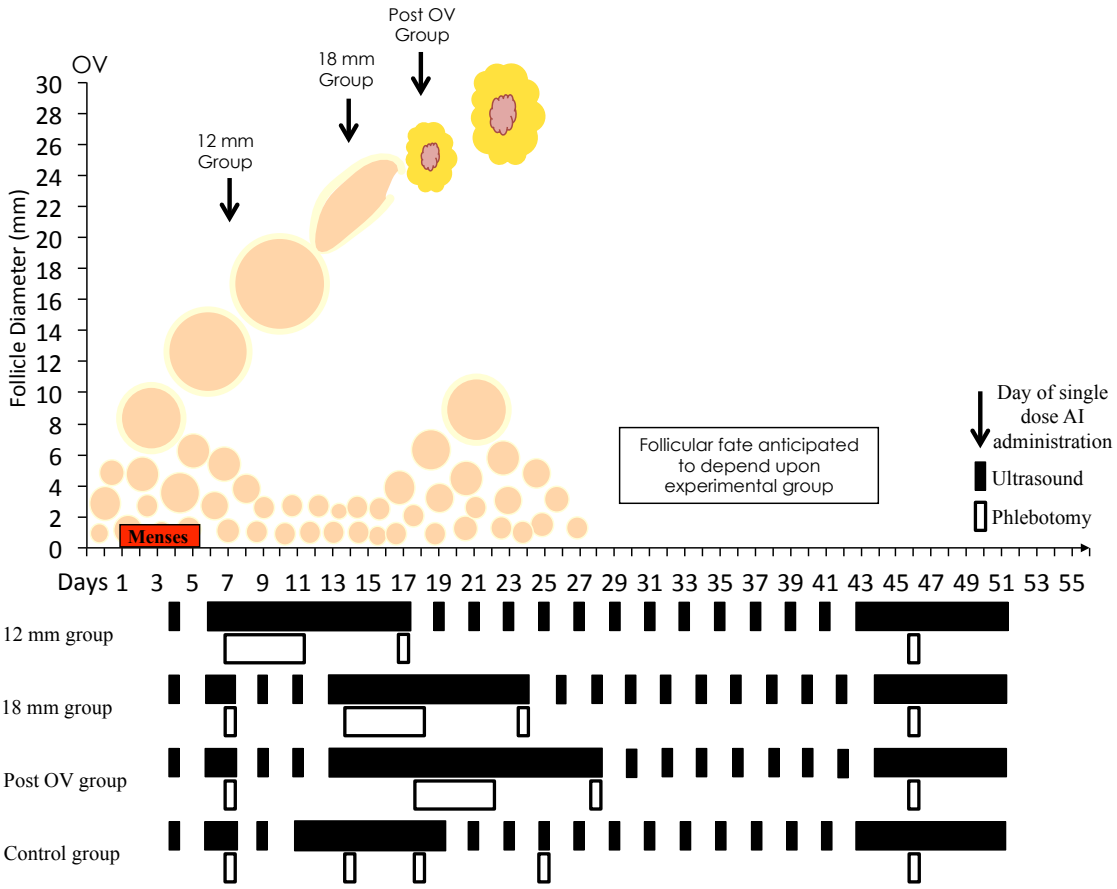

Figure 5.1: Schematic diagram of the study protocol.

Ultrasonography

Ovarian follicular and endometrial development were evaluated using transvaginal ultrasonography (Ultrasonix RP, Ultrasonix, Inc., Vancouver, BC). One individual performed approximately 96% of the examinations (HCMA). Three other investigators (TGBS, RAP, HVB) performed examinations when the primary ultrasonographer was unavailable. All examinations were conducted in accordance with standard operating procedures for ultrasonographic imaging at the Women's Health Imaging Research Laboratory.

Ultrasonographic scanning began on day 4 of the menstrual cycle (day 1 = first day of menses). Scans were performed every other day thereafter until randomization status was met. Following administration of letrozole, ultrasound examinations were performed daily for a minimum of 11 days and were continued daily until the fate of the dominant follicle was determined (i.e., regression, formation of an anovulatory follicular cyst, hemorrhagic anovulatory follicle (HAF), luteinized unruptured follicle (LUF), or OV). After determination of follicular fate, ultrasound examinations then returned to an every other day schedule until the dominant follicle of the next ovulatory wave reached 15 mm ± 1 mm. Daily examinations then resumed until the subsequent OV.

Diameters of all follicles ≥ 2 mm were tabulated at each ultrasound examination. The average of the mean follicle length and width in the transverse and sagittal planes was used as the mean follicle diameter for all follicles > 10 mm. The dominant follicle was defined as the follicle ≥ 10 mm that surpassed other follicles in the cohort by ≥ 2 mm in diameter. A co-dominant follicular wave was defined as a wave with 2 or more dominant follicles. Ovulation was defined as the disappearance of a follicle ≥ 12 mm in diameter detected ultrasonographically the previous day followed by the subsequent visualization of a luteal structure (152, 159).

Endometrial thickness and pattern were also recorded during each ultrasound examination as previously described (262, 279). Endometrial thickness was defined as the distance from the anterior stratum basalis-myometrial junction to the posterior stratum basalis-myometrial junction measured in the mid-sagittal plane at the largest dimensions of the fundal aspect of the uterus. Endometrial echotexture was assessed each scan as either an M, A, B, C, or D pattern.

Blood Sampling

All participants had blood drawn to measure serum concentrations of E_2, P_4, FSH and LH at specific time points throughout the study. Beginning on the day of treatment, blood was drawn daily for 5 days to measure changes in endocrine concentration following treatment. If the fate of the extant follicle was not determined after 5 days, blood was drawn every other day until follicle fate was determined. A subsequent blood draw was performed 5 to 9 days after the determination of dominant follicle fate to confirm follicle fate with P_4 levels. A final blood sample was taken when the subsequent pre-ovulatory follicle reached 18 ± 1 mm. Participants in the control group had a single blood sample drawn at the physiologic times (12 mm follicle, 18 mm follicle, 24 hours post OV) when treatment group participants would have administered treatment. Control group participants also had a final blood sample taken when the subsequent pre-ovulatory follicle reached 18 ± 1 mm.

Blood samples were collected into a 7 mL tube and allowed to coagulate for 30 to 45 minutes (min) at room temperature before centrifugation for 30 min at 700 x g. Serum was isolated and stored at -20°C until all participants had completed the study.

Daily Events Charts

All volunteers were provided a diary to record menstrual patterns, concomitant medication and any adverse events that occurred during the study.

Imaging Data Analysis

The identity and non-identity methods were used to characterize changes in follicle diameter during the study (90, 280). The non-identity method requires sorting all follicles ≥ 2 mm in descending order of diameter for left and right ovaries for each woman at each study visit separately. The size sorted follicles in left and right ovaries were then graphed over time. The identity method involved sketching the sizes and locations of all follicles ≥ 2 mm in

each ovary immediately after each scan (18, 271, 277, 281). The day-to-day identities of individual follicles were determined retrospectively using the size and anatomic location of the ovary with respect to the uterus as reference points. The diameter profiles of individual follicles that grew ≥ 8 mm over time for each woman were graphed (18). Mean growth profiles of the dominant and first subordinate follicles were graphed for each study group. Mean endometrial thickness profiles over the course of the study were also plotted for each group.

Hormone Assays

Sequential competitive fluorescence immunoassays (Immulite™, Siemens Healthcare Diagnostics Inc., Tarrytown, NY) were performed to measure serum FSH and LH levels. Sequential radioimmunoassays were performed to measure serum E_2 and P_4 concentrations. All assays were conducted at Prairie Diagnostic Services at the University of Saskatchewan. Minimal detectible limits were: FSH (0.1 mIU/mL), LH (0.1 mIU/mL), E_2 (1.4 pg/mL), and P_4 (0.02 ng/mL). Intra-assay coefficients of variation for control measurements of secondary antibodies were as follows: FSH (low = 3.0%, medium = 2.9%, high = 1.6%), LH (low = 2.6%, medium = 2.4%, high = 1.6%), E_2 (low = 2.4%, medium = 10.9%) and P_4 (low = 3.9%, medium = 6.3%, high = 7.6%).

Statistical Analyses

Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). Descriptive statistics for single point measurements were analyzed among groups using one-way ANOVA. Hormone concentrations on day of treatment were compared between groups using t-tests. Changes in hormone concentration (E_2, FSH, and LH) over time were analyzed using repeated measures ANOVA (PROC MIXED). Endometrial thickness, endometrial pattern and intervals from treatment to OV were compared using Kruskal-

Wallis ANOVA. Significance was set at $P < 0.05$. Results are expressed as the mean ± SEM. The magnitude of the drop in estradiol was defined by the equation ([E_2 day of treatment]–[E_2 day of nadir])/(day of nadir – day 1).

Results

The age, BMI, and waist-hip ratio (WHR) of participants were similar among groups ($P > 0.3$; Table 5.1). No adverse events were observed in any of the groups.

Table 5.1: Descriptive statistics for age (years), BMI (kg/m^2), and WHR (mean ± SEM) of participants.

Experimental Group	N	Age (years)	BMI (kg/m^2)	WHR
12 mm	10	24.80 ± 1.92	25.36 ± 0.80	0.75 ± 0.01
18 mm	10	28.00 ± 1.58	24.54 ± 1.12	0.76 ± 0.02
Post OV	10	24.00 ± 1.58	24.80 ± 1.40	0.75 ± 0.01
Control	11	25.18 ± 1.76	25.41 ± 1.12	0.79 ± 0.02

Follicular Dynamics

The mean diameter profiles of the dominant and first subordinate follicles of the ovulatory wave and the largest and second largest follicles of the first anovulatory wave for each experimental group are shown (Figures 5.2 to 5.5). The dominant follicles in all study groups ovulated. Ovulation was confirmed by ultrasonographic observation of a CL and a subsequent rise in P_4 on days 5-9. In the 12 mm group, 3 of 10 (30%) women developed co-dominant follicles, one follicle ovulated and the other regressed (n = 1) or double OV (n = 2) occurred. One of 10 (10%) women in the 18 mm group developed co-dominant follicles, one follicle ovulated and the other regressed. In the control group, one woman (18%) developed co-dominant follicles, one follicle ovulated and the other developed into a HAF. This participant was excluded from hormonal analysis.

The day of treatment and the interval from treatment to OV differed among treatment groups ($P < 0.0001$; Table 5.2). The day of treatment was earlier in the 12 mm

group and later in the 18 mm and post OV groups based on physiology and experimental design. The 12 mm group peak follicular diameter was larger compared the 18 mm and control groups ($P < 0.04$). The peak follicular diameters of the 12 mm and post-OV groups did not differ ($P = 0.1251$). Peak follicular diameter did not differ between the 18 mm, post-OV and control groups ($P > 0.5$). The interval from treatment to OV was longest in the 12 mm group and shortest in the 18 mm group. The interval from treatment to OV in the post OV group was negative because participants were administered treatment 24 hours following OV. There were no differences among study groups for follicular growth rate ($P > 0.1$; Table 5.2). Daily changes in dominant follicle growth are shown (Figure 5.6).

The interval from detection of an 18 mm follicle to OV was shorter in the 18 mm treatment group than in the control group (41.78 ± 13.12 h vs. 72.54 ± 34.92 h, respectively; $P = 0.006$). The interval from treatment to new follicular wave emergence was longer in the 12 mm group (7.7 ± 6.0 d) compared to the 18 mm and post OV groups (1.3 ± 2.8 d, -0.5 ± 3.0 d, respectively; $P = 0.0001$). The 12 mm, 18 mm, post OV and control groups had similar intervals from OV to new follicular wave emergence (0.4 ± 4.5 d, -0.2 ± 2.5 d, 0.5 ± 3.0 d, 0.8 ± 3.2 d, respectively; $P = 0.925$).

Table 5.2: Mean follicular end points (mean ± SEM) among experimental groups.

Experimental Group	N	Day of treatment (d)	Peak diameter (mm)	Follicular growth rate (mm/d)	Interval from treatment to OV (d)
12 mm	10	9.2 ± 0.4^{a}	23.2 ± 1.0^{a}	1.5 ± 0.1^{a}	7.4 ± 0.6^{a}
18 mm	10	14.5 ± 1.1^{b}	19.8 ± 0.6^{b}	1.3 ± 0.1^{a}	1.9 ± 0.2^{b}
Post OV	10	17.9 ± 0.8^{c}	$20.7 \pm 1.3^{a,b}$	1.5 ± 0.1^{a}	-1.0 ± 0.0^{c}
Control	11	NA	19.8 ± 1.4^{b}	1.2 ± 0.1^{a}	NA
P-value		0.0001	0.1266	0.1809	0.0001

All comparisons are within columns. Values with different superscripts indicate differences among experimental groups ($P < 0.05$). [a-c] Comparisons among experimental groups.

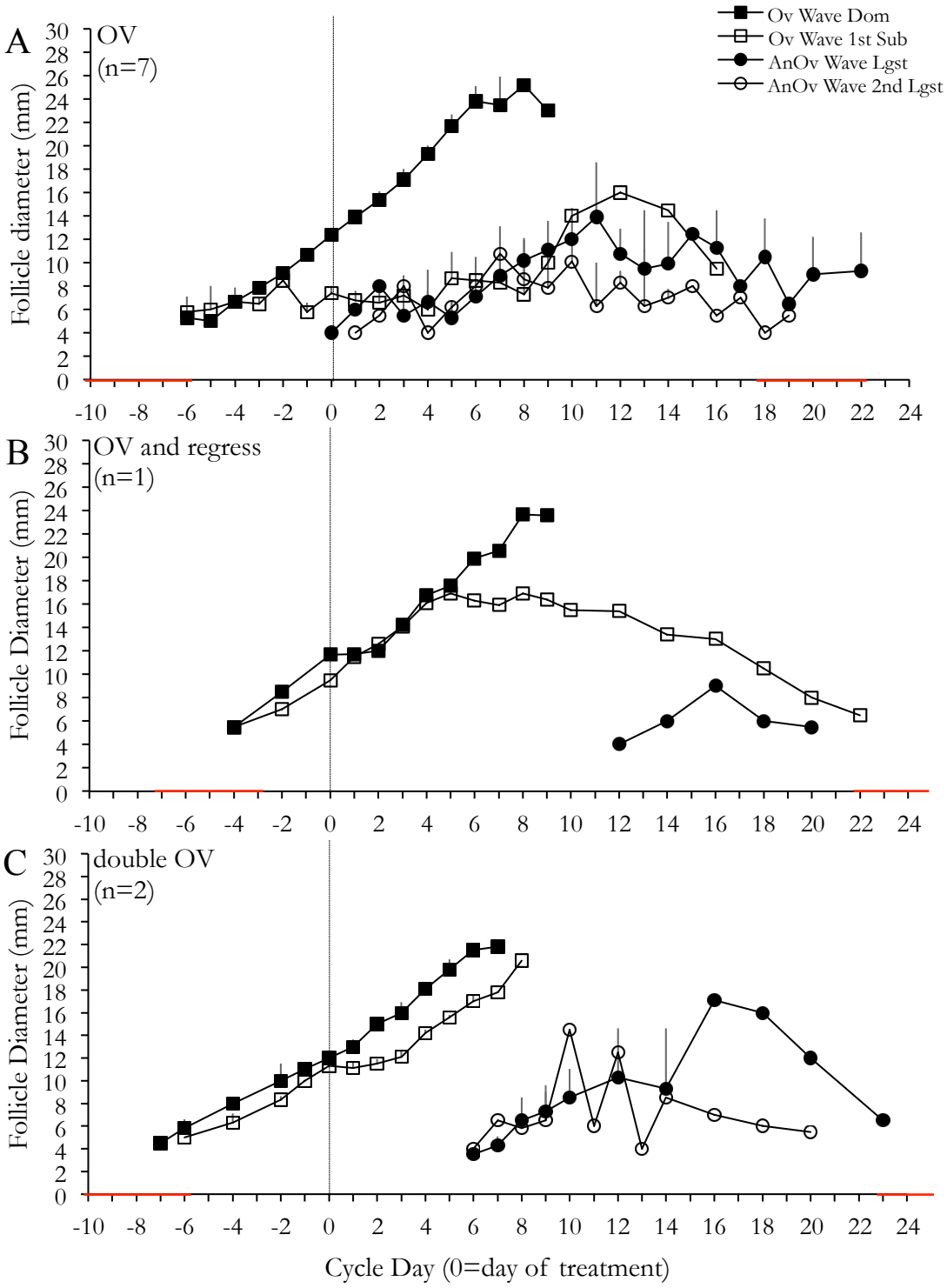

Figure 5.2 a-c: Mean follicle diameter profiles of the 12 mm group. Participants with follicles that ovulated (A), ovulated and regressed (B), and double ovulated (C) are shown separately. Data are shown for one inter-menstrual interval centralized to the day of treatment at 12 mm. The ovulatory wave dominant (▪) and 1st subordinate (▫) follicles and the anovulatory wave largest (•) and second largest (○) follicles are shown.

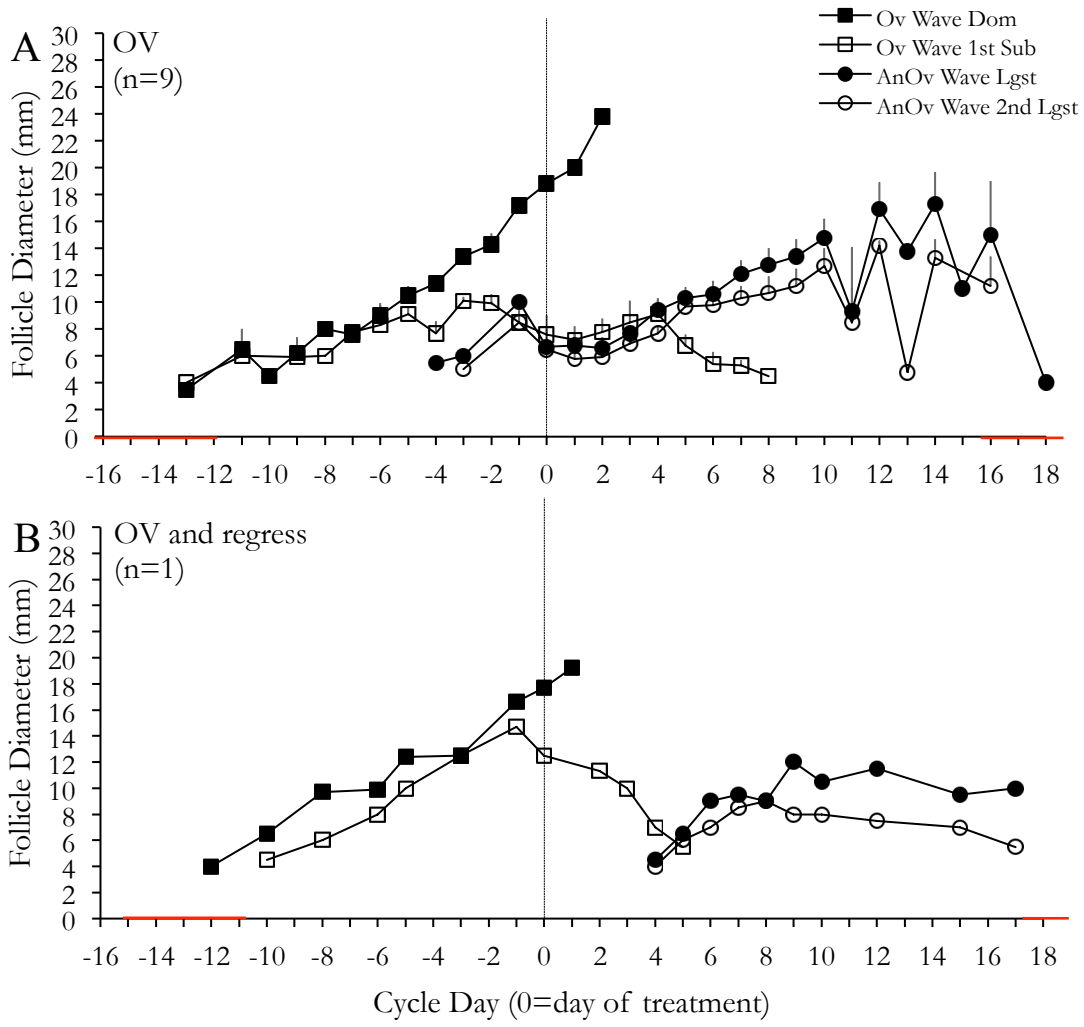

Figure 5.3 a & b: Mean follicle diameter profiles of the 18 mm group. Participants with follicles that ovulated (A) and ovulated and regressed (B) are shown separately. Data are shown for one inter-menstrual interval centralized to the day of treatment at 18 mm. The ovulatory wave dominant (▪) and 1st subordinate (▫) follicles and the anovulatory wave largest (●) and second largest (○) follicles are shown.

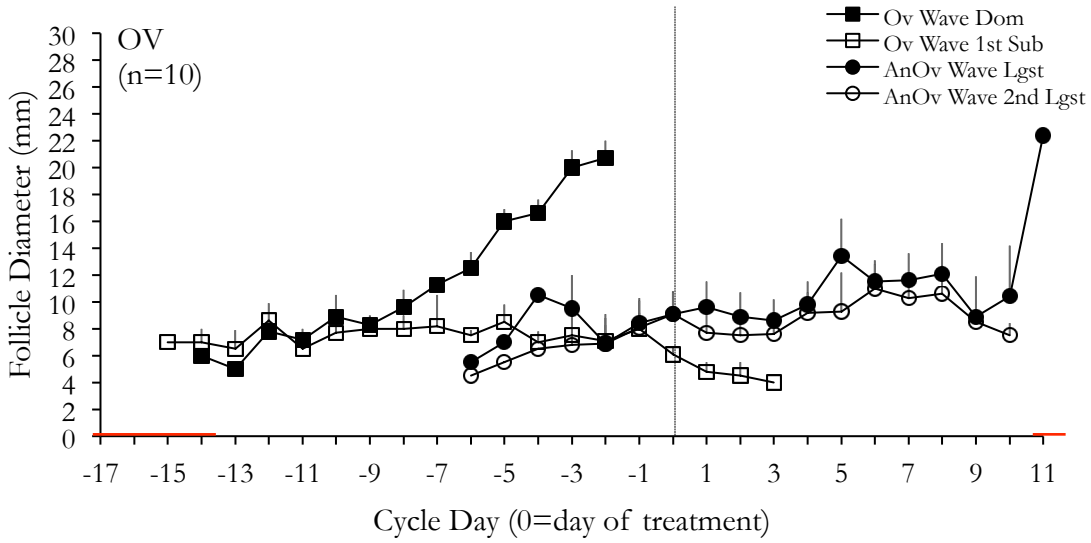

Figure 5.4: Mean follicle diameter profiles of the post-OV group. Data are shown for one inter-menstrual interval centralized to the day of treatment 24 hours after observation of OV. The ovulatory wave dominant (▪) and 1st subordinate (▫) follicles and the anovulatory wave largest (•) and second largest (○) follicles are shown.

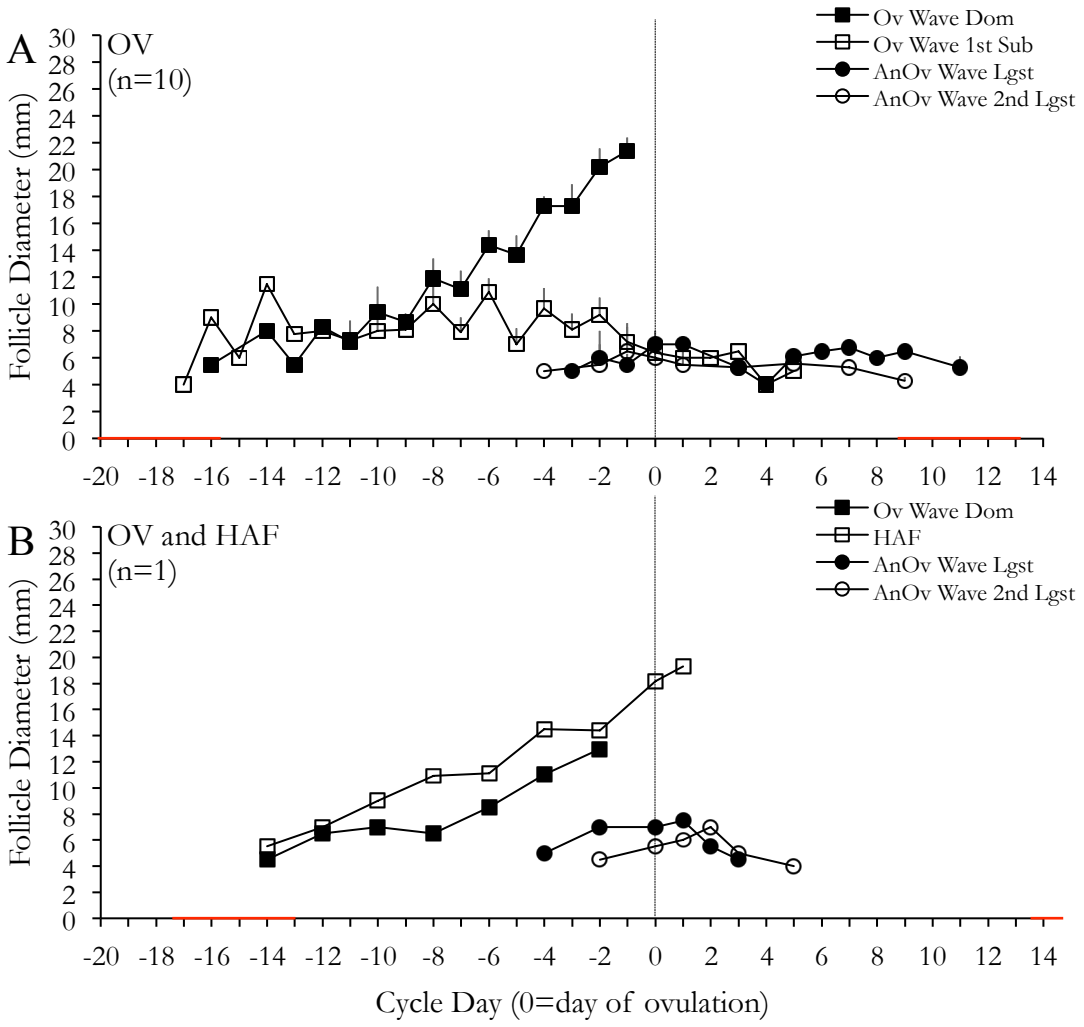

Figure 5.5 a & b: Mean follicle diameter profiles of the control group. Participants with follicles that ovulated (A) and ovulated and formed a HAF (B) are shown separately. Data are shown for one inter-menstrual interval centralized to the day of OV. The ovulatory wave dominant (▪) and 1st subordinate (▫) follicles and the anovulatory wave largest (•) and second largest (○) follicles are shown.

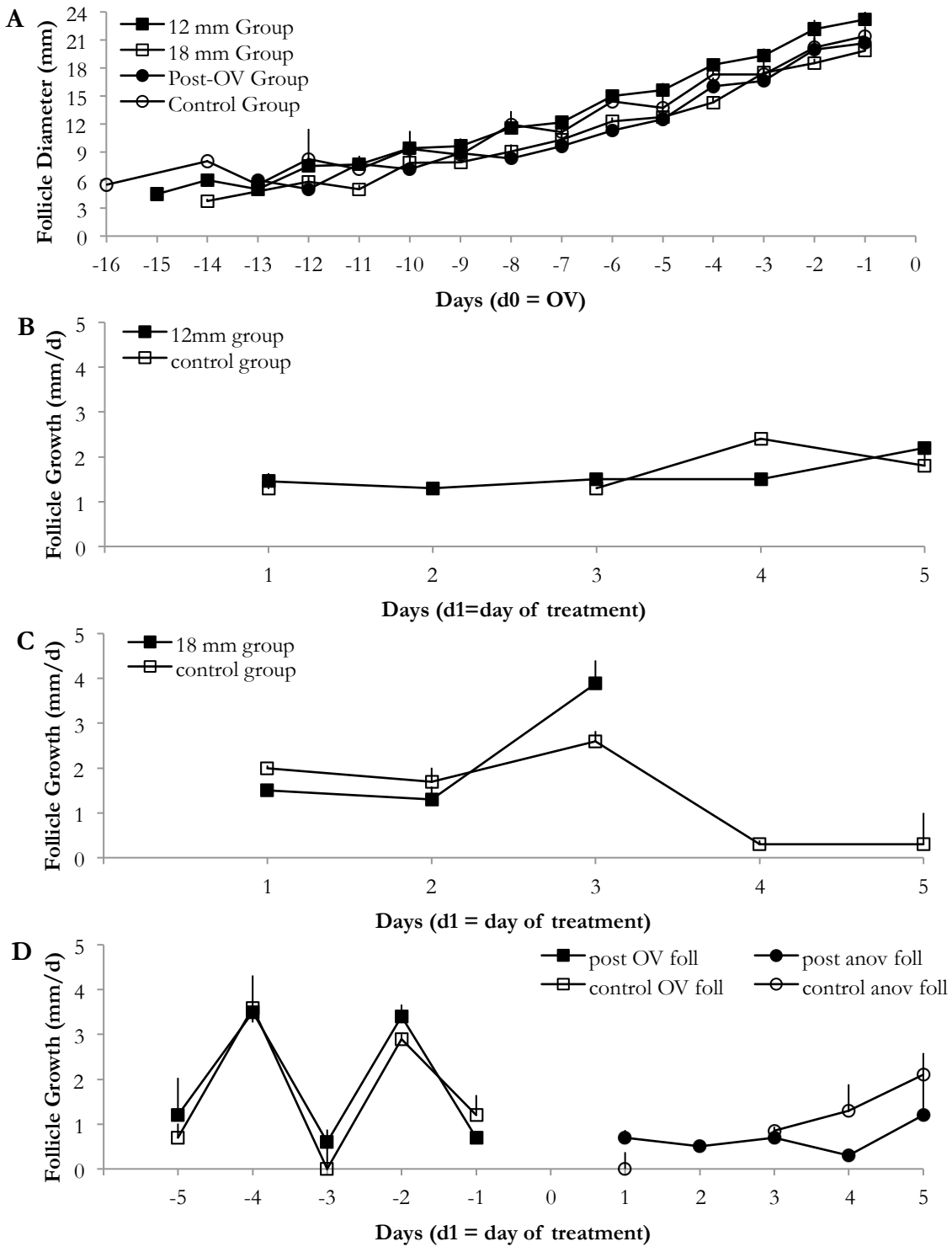

Figure 5.6 a-d: Dominant follicle growth over the follicular phase centralized to OV (A). Daily differences in dominant follicle growth centralized to day of treatment (day 1) for the 12 mm (B), 18 mm (C), and post-OV (D) groups. Daily differences in dominant follicle growth are compared to daily differences in dominant follicle growth in the control group centralized to the respective treatment initiation size.

Circulating Hormone Concentrations

The E_2 concentrations between each treatment group and the control group assessed at the same follicle diameter did not differ ($P > 0.25$; Table 5.3). The pattern of change in plasma E_2 concentration over 5 days beginning on the day of treatment differed among the 3 treatment groups ($P < 0.0001$; Figure 5.7). The 18 mm group exhibited higher ($P < 0.0001$) E_2 concentrations on the day of treatment attributed to the presence of a pre-ovulatory dominant follicle. Following treatment E_2 concentrations decreased in all treatment groups ($P = 0.0004$). The 18 mm group E_2 levels reached baseline on day 3 and remained at baseline until day 5 ($P = 0.8563$). The 12 mm group E_2 levels increased ($P = 0.01$) from day 2 to day 5. The magnitude of the initial drop in E_2 concentration in the 18 mm group was larger than in both the 12 mm and post OV groups ($P = 0.04$; Figure 5.8).

Table 5.3: Mean E_2, FSH, and LH concentrations on the day of treatment in each treatment group compared to the control group at the same respective time in the menstrual cycle.

	E_2 (pg/mL)	FSH (mIU/mL)	LH (mIU/mL)
12 mm group	40.33 ± 3.94^{a}	5.61 ± 0.37	6.1 ± 0.50
Control group at 12 mm	53.05 ± 13.23^{a}	NA	NA
18 mm group	131.9 ± 12.06^{p}	6.72 ± 1.13	21.46 ± 6.02^{p}
Control group at 18 mm	160.2 ± 10.19^{p}	NA	17.79 ± 4.65^{p}
Post OV group	66.53 ± 10.19^{t}	5.05 ± 0.84^{t}	7.43 ± 1.53^{t}
Control group post OV	49.77 ± 13.87^{t}	4.17 ± 0.42^{t}	7.04 ± 1.18^{t}

All comparisons are within columns and physiologic time. Values with different superscripts indicate differences ($P < 0.05$). [a] Comparisons between 12 mm and control groups. [p] Comparisons between 18 mm and control groups. [t] Comparisons between post OV and control groups.

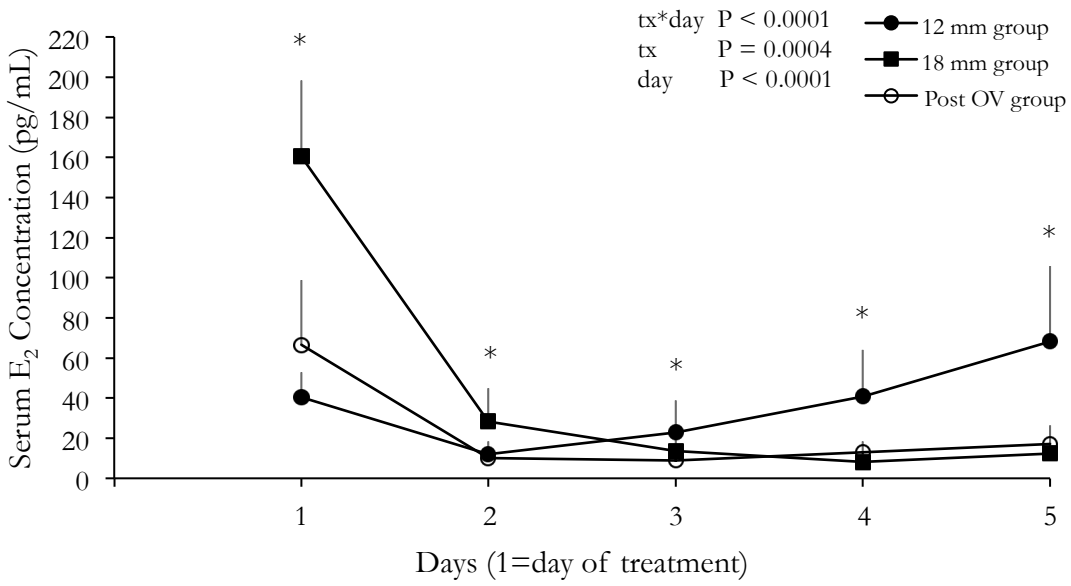

Figure 5.7: Mean changes in E_2 concentration over the experimental period in the treatment groups (day 1 = day of treatment). Differences among treatment groups are identified within days ($P < 0.05$). * Within day comparisons among experimental groups.

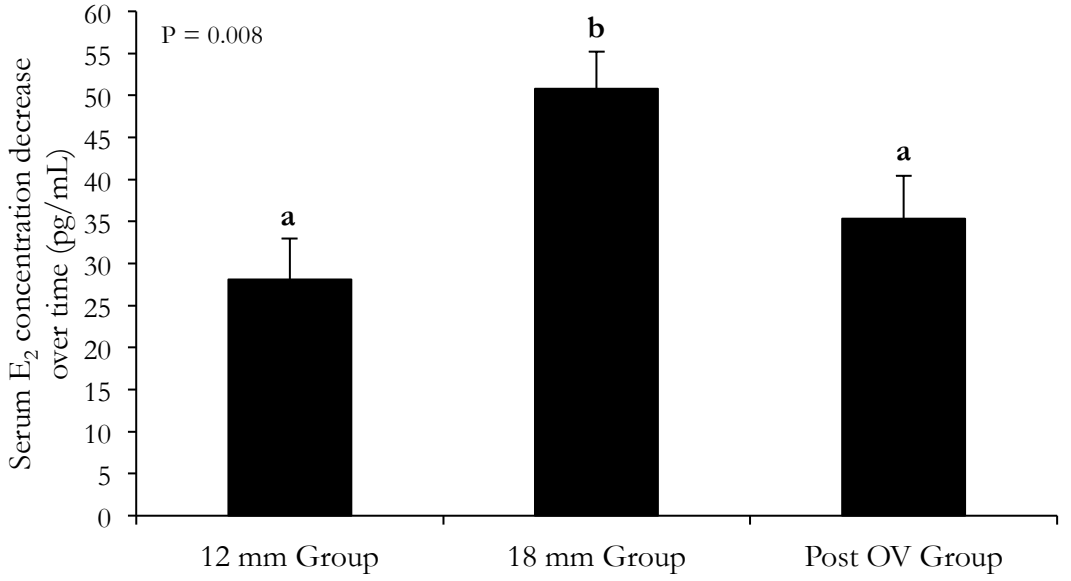

Figure 5.8: Drop in E_2 concentration from initial to nadir in the experimental period in the treatment groups. Differences among treatment groups are denoted by different superscripts. [a,b] Differences among experimental groups drop in E_2 concentrations to nadir.

Plasma FSH concentrations increased and subsequently decreased over the experimental period in all treatment groups ($P = 0.0014$; Figure 5.9). The treatment by day

interaction was attributed to the immediate FSH increase from day 1 to 2 in the 12 and 18 mm groups ($P < 0.0001$), while FSH levels in the post OV group first increased from day 2 to 3 ($P < 0.0002$). FSH concentrations in the 18 mm group were higher than those of the 12 mm and post OV groups ($P < 0.007$). FSH concentrations were highest in the 18 mm group on days 2, 4 and 5 following treatment ($P < 0.03$).

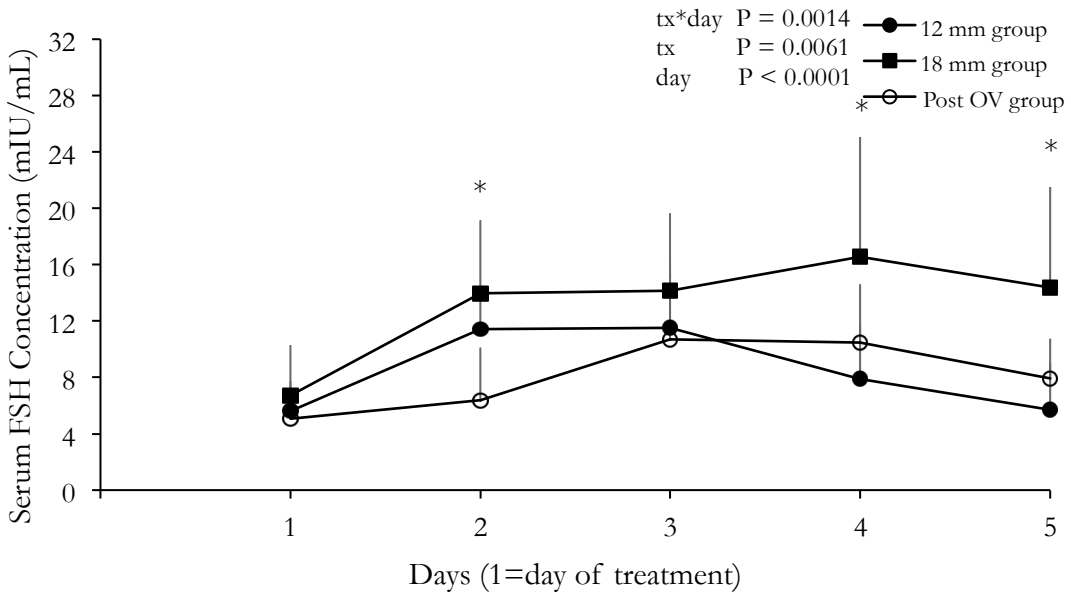

Figure 5.9: Mean changes in FSH concentration over the experimental period (day 1 = day of treatment) in the treatment groups. Differences among treatment groups are identified within days ($P < 0.05$). * Within day comparisons among experimental groups.

Following treatment, plasma LH concentrations in all treatment groups initially increased ($P = 0.0013$; Figure 5.10). LH concentrations then decreased to pretreatment levels by the end of the experimental phase ($P = 0.6329$). The mean plasma LH concentrations over the experimental period were higher in the 18 mm group versus the 12 mm and post OV groups ($P < 0.02$).

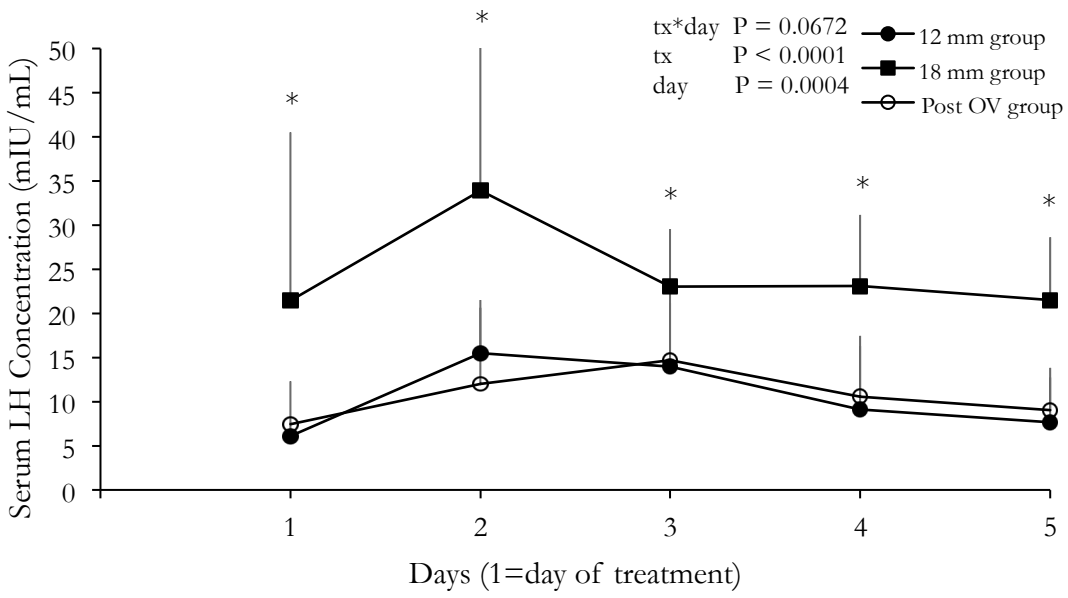

Figure 5.10: Mean changes in LH concentration over the experimental period (day 1 = day of treatment) in the treatment groups. Differences among treatment groups are identified within days ($P < 0.05$). * Within day comparisons among experimental groups.

The maximum concentrations of FSH and LH following treatment differed among treatment groups ($P < 0.03$; Figure 5.11). The 18 mm group had higher FSH and LH concentrations than the 12 mm and post OV groups ($P < 0.05$); maximum FSH and LH concentrations did not differ between the 12 mm and post OV groups ($P > 0.26$). The interval from the day of treatment to the day of maximum FSH was 2 days for the 12 mm group, 4 days for the 18 mm group and 3 days for the post OV group ($P = 0.0254$). The interval from the day of treatment to the day of maximum LH concentrations tended to be shorter for the 12 and 18 mm groups versus the post OV group ($P = 0.0833$).

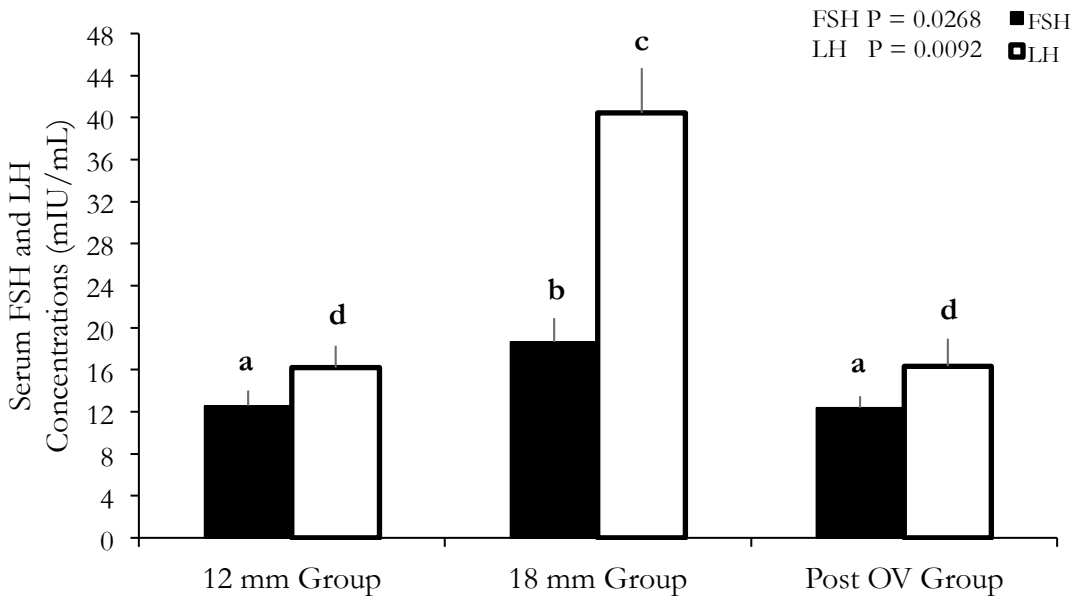

Figure 5.11: Mean maximum FSH and LH concentrations during the experimental period. Overall comparisons among treatment groups are denoted by different letters. [a,b] differences among treatment groups peak FSH concentrations. [c,d] differences among treatment groups peak LH concentrations.

Endometrial and Luteal Dynamics

Mean maximum endometrial thickness and pattern, and endometrial thickness and pattern at ovulation were not different among experimental groups ($P > 0.1124$; Figure 5.12). There were no differences in the interval from menses to OV, IOI, or IMI among experimental groups ($P > 0.2$; Table 5.4). Day 5-9 serum P_4 concentrations were 22.85 ± 5.46, 16.33 ± 1.84, 13.01 ± 1.41 and 24.12 ± 5.67 in the 12 mm, 18 mm, post-OV and control groups, respectively (mean ± SEM; $P = 0.1899$). Based on no differences among groups with regard to P4 levels, IMI and IOI there was no effect of treatment on CL formation. The 12 mm and 18 mm groups had longer intervals from treatment to menses than the post-OV group based on physiology ($P < 0.0001$; Table 5.4).

Table 5.4: Descriptive statistics for physiologically relevant intervals (mean ± SEM) among experimental group comparisons.

Experimental Group	N	Interval from menses to OV (d)	Inter-menstrual interval (d)	Inter-ovulatory interval (d)	Interval from treatment to menses (d)
12 mm	10	16.6 ± 0.5[a]	27.6 ± 1.1[a]	29.5 ± 1.6[a]	19.7 ± 1.3[a]
18 mm	10	16.4 ± 1.1[a]	29.6 ± 1.4[a]	26.9 ± 2.0[a]	16.1 ± 0.6[b]
Post OV	10	16.9 ± 0.8[a]	28.0 ± 1.0[a]	23.9 ± 3.0[a]	11.1 ± 0.9[c]
Control	11	17.2 ± 1.0[a]	28.5 ± 1.1[a]	29.0 ± 1.1[a]	NA
P-value		0.9284	0.6551	0.2014	0.0001

All comparisons are within columns. Values with different superscripts indicate differences ($P<0.05$). [a-c] Comparisons between experimental groups.

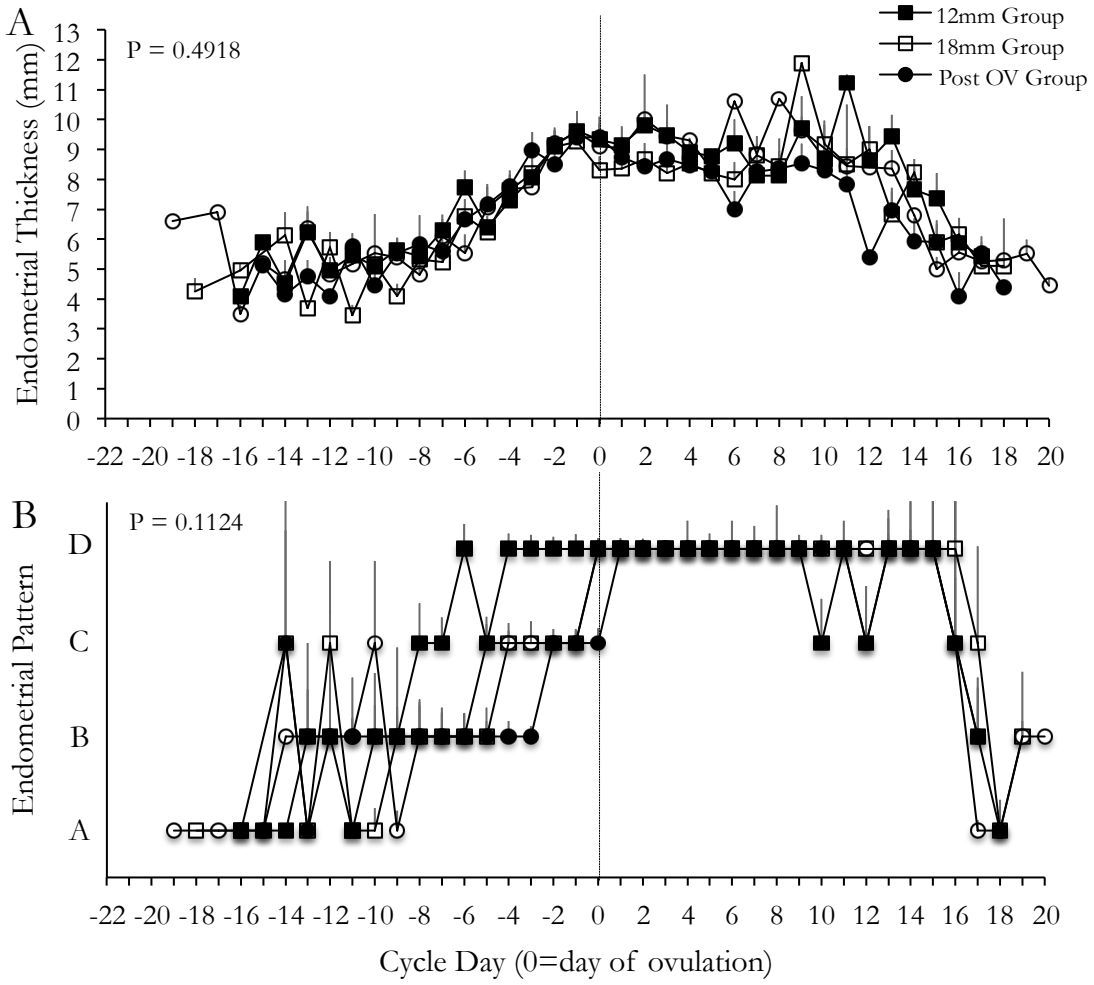

Figure 5.12 a & b: Mean profiles of endometrial thickness (A) and pattern (B). Women in all experimental groups, 12 mm (▪), 18 mm (▫), 24 hours post ovulation (•), and control (○), are identified. Data are shown for one inter-menstrual interval and are centralized to the day of ovulation. No significant effect of day observed for endometrial thickness or pattern.

Discussion

Our hypothesis that a single 20 mg dose of letrozole given at defined times of the natural menstrual cycle would cause ovulatory failure and failure of CL formation or impaired luteogenesis was not supported. Similarly, arrested endometrial development, emergence of a new follicular wave, and a shortened interval to menses were not observed. Our hypothesis that letrozole administered at defined times of the menstrual cycle resulted in a drop in E_2 and a rise in FSH was supported; however, our hypothesis that there would be no change in LH was not supported.

Treatment administered peri-selection of a dominant follicle (i.e., the 12 mm group) was provided to determine the effects of E_2 deprivation on pre-ovulatory follicle growth. In this group the selected follicle continued to grow an attained a larger pre-ovulatory size compared with the other pre-ovulatory treatment group (i.e., 18 mm group) and control group. When treatment was provided at an ostensibly pre-ovulatory size (i.e., the 18 mm group) there was no change in follicle diameter at OV compared to the control group. There was a tendency for the pre-ovulatory follicle diameter in the 12 mm group to be larger compared to the post-OV group. The trend of a larger follicle in the 12 mm group suggests that there is a compensatory mechanism for the acute drop in E_2 in the mid-follicular phase. We postulated that this compensatory mechanism was a result of a rise in FSH and LH that was observed following AI treatment. However, with our small sample size we were unable to conclude that AI treatment effects pre-ovulatory follicle diameter.

Treatment in the follicular phase did not alter the growth profile of the dominant follicle in the present study. This is contrary to results reported in domestic farm animals. In the bovine species, a single dose of letrozole after follicle selection prolonged the interval from treatment to OV (277, 278, 282). To date there has been no evaluation of the effects of

AI on follicle growth profiles when administered prior to dominant follicle selection. Dominant follicle selection during natural human menstrual cycles is thought to occur at a mean diameter of approximately 10 mm (34). Initiation of AI treatment prior to selection as used in ovarian stimulation had a different effect on extant follicle development. Use of AI prior to selection may have more effectively released FSH secretion stimulating further growth of the extant follicular wave; however, studies did not measure changes in FSH during treatment (44, 45, 48, 50, 51, 150, 182, 183, 201, 206-209, 215, 283-287). In retrospect, it would have been useful to include a treatment group in which women received letrozole after day 3 and before the largest follicle attained a diameter of 10 mm. The use of this strategy may provide insight into the roles of FSH concentration and E_2 during selection.

Follicle development in all treatment groups continued despite a drop in E_2 production following letrozole treatment in women. Decrease in E_2 was observed within 24 hours of letrozole treatment in all women in our treatment groups, supporting our hypotheses. These findings contrast those previously documented in the bovine species, where increasing mean plasma E_2 levels were observed for 4 days following the day of letrozole treatment. The E_2 increase was associated with a simultaneous increase in follicle diameter (282). In the present study, only women the 12 mm group had an increase in E_2 level following letrozole use in the follicular phase. Estradiol levels were not available for comparison during the same time frame in the control group. An increase in E_2 concentration began 2 days after treatment and was attributed to the continued growth of the pre-ovulatory dominant follicle. The absence of a rise in E_2 following 18 mm and post-OV group treatments can be attributed to a physiologic drop in E_2 secretion following OV, as documented in previous studies of natural cycle OV (3).

A rise in FSH following treatment observed in all treatment groups in the present study supported our hypothesis. Treatment with letrozole in the luteal phase in women resulted in a FSH rise 7 days after treatment (288). However, increases in FSH concentration in women contrast with the lack of FSH change in response to letrozole treatment previously reported in the bovine species (277, 278, 282). The pattern of FSH rise in all three treatment groups differed; however, all three groups exhibited a rise in FSH for the 4 days following treatment to typical mid-follicular phase levels. In natural menstrual cycles, the duration of the FSH rise at wave emergence is short due to the negative feedback system of E_2 and inhibin B. This mechanism is postulated to ensure monovulation (106). The 4-day rise in FSH following letrozole treatment appears to elongate the FSH rise thus potentially allowing multiple dominant follicles to develop.

In the present study, women in the 12 mm, 18 mm and control groups developed co-dominant follicles. In the 12 mm group, 20% of the women ovulated 2 follicles during the treatment period, in contrast with the reported 4% natural occurrence of double OV (88, 159, 289, 290). Another participant in the 12 mm group developed a second dominant follicle which regressed. This observation is conceptually similar to a recent study in cattle wherein the 1st subordinate follicle of animals given AI in the follicular growth phase (d1-3) exhibited larger mean diameters (277). It was postulated that the extended FSH window in women allowed a subordinate follicle to be rescued from atresia. In both the 18 mm and control groups, a second dominant follicle grew but either regressed (10%) or resulted in ovulatory failure (9%). Ovulatory failure occurs in approximately 5% of cycles of regularly cycling women (291) and 10% of cycles in infertile women (260, 292). We did not see an increased incidence of ovulatory failure in this study.

Circulating LH concentrations reached maximum levels within 24 hours of treatment and returned to starting levels by the end of the experimental period in all groups. The rise in LH concentrations within 24 hours is consistent with reports of letrozole treatment in cattle (277, 278, 282). An increase in mean LH concentration in women has only previously been shown on day 6 of a 7 day low dose follicular phase letrozole treatment (293). A sustained elevation in LH following cessation of follicular phase AI treatment has not previously been reported in women (42-45, 47, 48, 196, 198, 203, 206, 208, 285, 293, 294). Most reports involving the use of AI in women are based on research in fertility patients where chemical and clinical pregnancy were the final outcome measures (42-45, 47, 48, 196, 198, 203, 206, 208, 285, 294). In these reports, LH concentration is measured only after hCG is given to induce OV (42-45, 47, 48, 196, 198, 203, 206, 208, 285, 293, 294). In bovine studies, it has been suggested that the prolonged elevation in LH following AI treatment in the follicular and luteal phases indirectly enhances dominant follicle development (277, 278, 282).

The failure of letrozole to interrupt follicle development in the 12 mm and 18 mm groups in the present study indicates a potential post-selection compensatory mechanism for an acute drop in estrogen production. The mechanism may involve an indirect enhancement of follicle growth by an increase in LH levels, as inferred from observations in the bovine model (277, 278, 282). In humans a post letrozole treatment increase in LH levels in concert with a similar increase in FSH levels may facilitate continued follicular development.

Limitations in our study design are related to the unexpected study outcomes that we observed. We hypothesized that letrozole treatment would result in ovulatory failure and hence did not anticipate that a need would arise for the evaluation of serial luteal phase P_4. The issues arising from the experimental design with respect to the control group blood collection were the biggest limitations of the study. Blood draws in the control group were

collected to coincide with the times of letrozole treatment at defined times: peri-selection, pre-ovulatory, and post-OV. This experimental design did not allow for adequate endocrine comparisons between the treatment and control groups during the experimental period. However, the optimal design facilitating comparison of the control group to the treatment groups during the experimental period would have required volunteers to have had blood collected daily for approximately 15 consecutive days. We were therefore limited by our inability to attract volunteers who would undergo daily blood sampling and transvaginal ultrasonography in this protocol. In addition, it was financially untenable to have a separate control group in this study for each treatment group.

In summary, letrozole treatment at follicle diameters of 12 mm or 18 mm or 24 hours post OV were associated with transient decrease in E_2 levels and elevated circulating FSH and LH concentrations for 4 days following treatment. The drop in E_2 concentration did not suppress dominant follicle growth or early CL development. These results were unexpected and provide an impetus for additional studies to elucidate the roles of E_2, LH, and FSH in the regulation of follicle and luteal development in women.

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Chapter 6: General Discussion

Men and women have been working to control fertility with many forms of contraception and methods to enhance fertility for thousands of years (295, 296). The available methods have been improved greatly because of the demand for safe, effective therapies. There are increasing numbers of women utilizing contraception to prevent pregnancy or assisted reproductive technologies to conceive (297, 298). Though the demand for manipulation and control of ovarian function is increasing, most women, as well as scientists and clinicians, do not completely understand the menstrual cycle (299). The ovary is the "master" gland of female reproductive function. An understanding of ovarian function is integral to comprehending reproductive function. The social stigma and personal embarrassment commonly associated with an inability to adequately control reproductive processes may prevent many individuals from becoming informed about their own reproductive physiology and possible contraceptive and fertility therapy choices.

Letrozole, a non-steroidal aromatase inhibitor (AI), is used as an adjuvant therapy for hormone sensitive breast cancer in post-menopausal women (300-302). Aromatase inhibitors work through systemic inhibition of aromatase to reduce the amount of circulating estrogen (178, 181, 275, 276, 300-304). In pre-menopausal women, AI have been administered in fertility treatments for the purpose of follicular stimulation, ovulation (OV) induction, and ovarian super-stimulation (51, 203, 206, 207). Letrozole in fertility therapies is administered before follicular dominance is manifest. Administration before follicular selection has been reported to have a stimulatory effect on ovarian function that is mediated by increased follicle stimulating hormone (FSH) secretion (286). The treatment goals for fertility therapy include follicle development, OV and clinical pregnancy.

It has been proposed that letrozole administration before follicle selection eliminates the negative feedback of estradiol (E_2) on the hypothalamus/pituitary axis (43, 46, 182, 197). Release of negative feedback induces a rise in circulating FSH concentrations (43, 46, 182, 197). This extends the growth phase of the follicles in the extant wave. However, the effects of AI on follicular dynamics in women do not appear to have been critically determined in any scientific reports. We hypothesized that AI treatment after follicle selection would have a suppressive effect on extant follicle development. Dominant follicle suppression could result in a rise in FSH secretion and subsequent emergence of a new follicular wave. We conducted an experiment to determine the effects of administering a single large administration of letrozole on follicular dynamics post selection (12 mm), peri-OV (18 mm), and post-OV (Chapter 6). The study reported in this thesis failed to show that a single large dose of letrozole at the specific points of post selection follicle growth would induce the regression of the dominant follicle and the emergence of a new follicular wave. Conversely, administration of AI peri-selection of a dominant follicle resulted in continued follicle growth, OV, a transient drop in E_2 concentrations and a prolonged rise in FSH and luteinizing hormone (LH) concentrations.

Contrary to our hypothesis, letrozole treatment did not result in extant follicle regression and new wave emergence. This finding is in contrast to reports of oral contraception (OC) initiation at 10 mm and 14 mm follicle diameters (34). Initiation of oral contraception (OC) at a follicular diameter of 10 mm (i.e., at selection) resulted in continued growth followed by regression (34). The observations from initiation of OC at 10 mm and 14 mm follicles are consistent with reports of follicle growth following AI treatment in heifers (277, 282). Administration of OC at a 14 mm follicle only resulted in OV in 36% of cases in women (34) versus OV in 100% of participants in the 12 mm group of the present

study. The differences in post selection follicle growth following temporary E_2 suppression or exogenous estrogen availability could result from distinct rescue mechanisms. The disparity between follicle growth patterns following OC initiation and AI treatment initiation are compelling reasons to have included a pre-selection AI treatment group. The differences in follicle growth, with or without E_2, and before or after selection indicate there are probably multiple mechanisms involved in rescuing follicles from initiating atresia.

Although E_2 production decreased following treatment, follicle growth continued and the dominant follicles in the immediate post selection (12 mm) and peri-OV (18 mm) treatment groups ovulated. Letrozole treatment did not affect the growth rate of the pre-ovulatory follicle or interval from menses OV (Chapter 6). The growth rates of all treatment groups follicles were similar to reported follicle growth rates in natural, oral contraceptive and fertility stimulation cycles (261). The lifespan of the dominant follicle was extended with a single dose and 3 day AI dosing administration in heifers (277, 282). The results are consistent with reported observations of follicle development following OC initiation at a 14 mm or 18 mm follicle diameter (34). The dramatic drop in E_2 with letrozole treatment and the exogenous rise in estrogen with OC initiation were both associated with increased LH levels. However, in the absence of E_2 it appears that a rise in circulating LH concentrations may play a more prominent role in follicle survival.

Some women treated with letrozole early in the follicular phase (treatment initiation on day 3 of the menstrual cycle) grew two dominant follicles to ovulatory status (45, 48, 201, 206, 208, 214, 215, 287, 294, 305). Treatment immediately post selection in our study resulted in double OV in 20% of the treatment group. The mechanism may be due to LH responsiveness in subordinate follicles at the time of selection (306). Synchronous development of 2 follicles also was observed in heifers following post-OV AI treatment

(277). In the bovine model it has been shown that if the dominant follicle is removed a subordinate follicle is capable of assuming dominance (307). Therefore, some subordinate follicles may be slightly LH responsive; an elevation in LH post treatment may have stimulated the growth of the largest subordinate follicle. Another mechanism that could have resulted in the survival of the largest subordinate follicle is an increase in follicular fluid androgen levels following AI administration. Androstenedione levels increased in the follicular fluid of Rhesus monkeys treated with an AI (150) and circulating androgen levels increased in women given letrozole treatment (214, 293). In bonnet monkeys, increases in androgen levels amplified FSH receptor (FSHr) numbers and increased the sensitivity of the developing follicle to available circulating FSH (196). Though androgen levels were not tested in the serum collected in this thesis, an increase in androgen levels following letrozole treatment would not be unexpected and would allow for increased FSHr and increased sensitivity of all viable follicles to the rise in circulating FSH.

Letrozole treatment at different biologically important times in follicle growth has similar effects on LH secretion but differential effects on FSH secretion. The peak in LH occurred quickly in all treatment groups, while the peak in FSH occurred on a different day in all treatment groups. The immediate reasons for the difference in response remains poorly elucidated; however, induction of an E_2 decrease at varying stages of follicle growth and the subsequent increases in androgens and P_4 could account for the differences in the pituitary response. The prevailing convention in AI treatment for ovulation induction in fertility therapy is to evaluate gonadotrophins before treatment and after human chorionic gonadotropin (hCG) injection (45, 198, 202, 204, 206, 208, 308). Elevated LH concentrations, but no elevation in FSH concentrations, were observed on the last day of a 5 day pre-selection letrozole treatment (305). On day 6 of a 7 day pre-selection treatment

regimen, an overall higher mean LH concentration was found in letrozole treated women compared to controls (293). Thus, even before dominant follicle selection, AI treatment affected LH and FSH secretion differentially.

A rise in LH secretion the day following letrozole treatment was observed in all treatment groups. In the 18 mm treatment group the rise in LH resulted in OV of the follicle at a mean of 42 hours after treatment compared with 72 hours in the control group. The consistent interval observed from treatment to OV may be interpreted to mean that there is a potential use for AI in inducing an LH surge. Administration of a single dose of AI at pre-ovulatory follicle diameters could reduce fertility patient exposure to exogenous hormones by reducing the need for FSH, hCG, or GnRH agonist/antagonists. Following AI treatment in the 12 mm group a natural LH surge occurred to induce OV of the pre-ovulatory sized follicle. A natural LH surge at pre-ovulatory follicle size has also been shown to occur following AI treatment administered before follicle selection (196, 204, 305, 308). The observation of a continued follicle growth following a single treatment of letrozole at a 12 mm follicle in normally cycling women indicates a new potential treatment for infertility patients. Combining 2 large doses given on day 3 of the menstrual cycle and at the observance of a 12 mm follicle is a potential way to reduce the need for exogenous FSH injections and prevent ovarian hyperstimulation.

There does not appear to be an effect of AI on endometrial morphology. Following early single dose and 5 day dosing schemes, endometrial thickness and pattern did not differ from controls mid-cycle (198, 202, 204, 206, 294, 309). Mid-cycle there were no difference in endometrial pattern or thickness in the present study (Chapter 6). There have been reports of the endometrium of letrozole treated women being thinner in the mid-follicular phase but having recovered to levels of non-letrozole treated patients by the time of hCG

administration (309). Thicker mid-cycle endometrial linings have been reported in women taking a 5 day treatment of 7.5 mg/day compared to women taking 2.5 or 5 mg/day (208). In our study, the endometrial thickness of all experimental groups did not differ throughout the menstrual cycle. However, women in the 12 mm treatment group reached a D pattern endometrial morphology earlier than women in the other 3 experimental groups (Chapter 6). Thus, there appears to be a mechanism that allows the endometrium to compensate for a decrease in E_2 throughout the menstrual cycle.

Letrozole affected ovarian function in an unexpected way. Inhibition of E_2 with an AI post-selection of a dominant follicle increased release of FSH and LH. The increase in LH concentrations resulting from AI treatment may have aided continued growth of the extant dominant follicle and allowed OV to occur without the need for hCG treatment when a pre-ovulatory follicle diameter was reached. Further, the rise in LH following administration of AI at a pre-ovulatory follicle diameter stimulated OV earlier than anticipated. We have proposed a new mechanism of action for AI on ovarian function following DF selection involving increased endogenous levels of LH and FSH. Aromatase inhibitors represent a potentially safe way to reduce the exposure of women to exogenous steroids and gonadotropins during fertility therapy.

Chapter 7: General Conclusion

The result of the experiment included in this thesis and the related literature have led to the conclusion that a single 20 mg dose of letrozole did not cause ovulatory failure or failure of CL formation by suppressing E_2 production. Subsequently, letrozole treatment did not arrest endometrial development, shorten the interval between menses, or initiate new follicular wave emergence. The results of our study are supportive of an alternative hypothesis regarding the control of folliculogenesis through a transient increase in LH secretion following Letrozole treatment presented in the literature (277, 278, 282).

The specific conclusions of this thesis are:

1. Letrozole given once orally at a dose of 20 mg in pre-menopausal, norm-ovulatory women does not reduce dominant follicle growth or cause ovulatory failure regardless of the stage of development at treatment;
2. A single, oral dose of 20 mg of letrozole in pre-menopausal, normo-ovulatory women did not alter the formation of the CL as observed by the similar mean P_4 levels, IMI and IOI;
3. Letrozole treatment given as a single oral dose of 20 mg in pre-menopausal, normo-ovulatory women causes a transient decrease in E_2 and increased circulating FSH and LH concentrations regardless of the stage of follicle development at treatment; and,
4. Higher circulating LH concentration following a single, 20 mg oral dose of letrozole in pre-menopausal normo-ovulatory women can stimulate continued growth of the dominant follicle.

Chapter 8: References

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